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CONTENTS OF THIS ISSUE

Partially Hydrolyzed Dextran 177
Alice G. Renfrew and *L. H. Cretcher*
A Quantitative Assay Method for Pyrogens 179
Walther H. Ott
The Preparation and Composition of Groundnut Cake, *Arachis Hypogea* (Peanut) Hydrolysate 184
R. Raghunandana Rao and *P. R. Venkataraman*
Influence of Amphetamine Sulfate on Cerebral Metabolism and Blood Flow in Man 186
Benedict E. Abreu, Grant W. Liddle, Arthur L. Burks, Violette Sutherland, Henry W. Elliott, Alexander Simon and *Lester Margolis*
Some Effects of Meperidine (Demerol) on Gastro-Enteric, Extra-Hepatic Biliary and Cardiovascular Activity 188
Raymond W. Pickering, Benedict E. Abreu, David F. Bohr and *William F. Reynolds*
A Preliminary Phytochemical Investigation of the Tannin Obtained from *Pinus caribaea* Morelet 192
Frank W. Bope and *Ole Gisvold*
A Phytochemical Investigation of the Kelps *Cymathaere Triplicata, Hedophyllum Sessile,* and *Egregia Menziesii* 196
Rob S. McCutcheon, Louis Arrigoni and *Louis Fischer*
Laboratory Assay of Clinically Effective Antiepileptic Drugs 201
Ewart A. Swinyard
Preliminary Report on the Volatile Oil of *Pycnanthemum Pilosum* Nutt 204
Victoria Chilquist, Robert Gilmour and *Paul Jaunke*
A Comparative Study of the Effect of Various Solutions on the Phosphorus Depletion of the Rat Tooth Using Radioactive Phosphorus 206
Albert E. Jarvis, John E. Christian, Leroy D. Edwards and *Glenn L. Jenkins*
Studies on Pharmaceutical Powders and the State of Subdivision. I. The Application of Low-Temperature Nitrogen Adsorption Isotherms to the Determination of Surface Areas 210
J. V. Swintosky, S. Riegelman, T. Higuchi and *L. W. Busse*
Effects of Surface-Active Agents on the Crystallinity of Carbowax Compounds 216
M. V. Nadkarni, Donald B. Meyers and *Louis C. Zopf*
A Critical Study of the Diffusion of Iodine and Sulfathiazole from Ointments 222
Laurence D. Lockie and *Joseph B. Sprowls*
The Antidiuretic Activity of the Posterior Pituitary and Its Quantitative Evaluation 227
K. M. Lindquist and *L. W. Rowe*
The Absorption and Penetration of Therapeutic Agents from a Carbowax Vehicle 231
Donald B. Meyers, N. V. Nadkarni and *Louis C. Zopf*
Book Reviews 234
Advertising ..... Cover pages 2, 3, 4 and pp. I–IV

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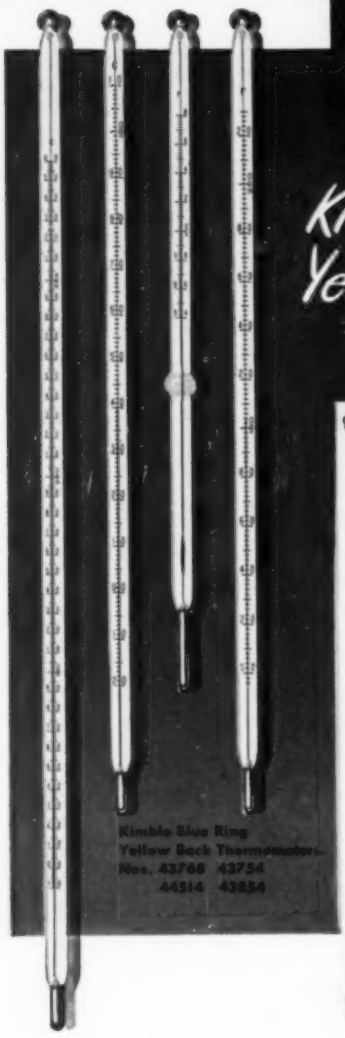

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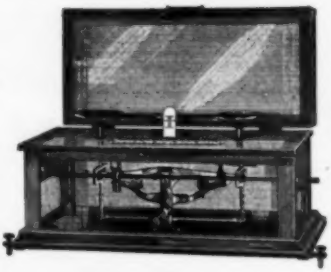

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Partially Hydrolyzed Dextran*

By ALICE G. RENFREW and L. H. CRETCHER

A high-molecular weight nontoxic colloid as a substitute for plasma in intravenous injection would find certain uses. Partially hydrolyzed dextran has been considered in this field (2). The experimental work presented is a study of the results of autoclaving dextran solutions in the presence of very dilute acid as a method for achieving mild hydrolysis. Viscosity measurements are reported. Fractionation with alcohol presented the problem of two-phase formation. After preliminary animal studies, it was not deemed advisable to continue the work with a view to using dextran on patients with nephrosis.

REPEATED efforts have been directed toward the search for a nontoxic colloid of high molecular weight to serve as a substitute for plasma in intravenous injection, or as a diuretic agent in the treatment of nephrotic edemas. A recent editorial in the *Journal of the American Medical Association* (1) provides a reminder that there still exists a need for plasma substitutes. During World War II the use of partially hydrolyzed polysaccharide dextran for intravenous injection was suggested by Grönwall and Ingelman (2) and this material has received clinical trial in 1000 cases in Sweden with special application to the treatment of shock and burn (2*d*).[1]

The present investigation is a study of one method which permits mild hydrolysis of dextran, i.e., the autoclaving of solutions of dextran in the presence of very dilute acid. The extent of hydrolysis was followed by measuring the decrease in viscosity of solutions subjected to autoclaving for various time intervals with water, with 0.01 *N* and with 0.001 *N* hydrochloric acid. Two studies of the fractionation of the partially hydrolyzed dextrans show that, for uses requiring rather uniform molecular size, fractionation would be necessary. Fractionation was somewhat complicated by the formation of two phases in approximately 40% ethanol.

The above hydrolytic procedures were more drastic than normal sterilization. However, a check was made of the effect of sterilization on the neutral, relatively dilute solutions to be considered for injection. In the sterilization of 5% solutions of degraded dextrans, no measurable change in viscosities was observed.

* Received Oct. 26, 1948, from the Department of Research in Pure Chemistry, Mellon Institute, Pittsburgh 13, Pa.

[1] Since the present paper was submitted, two articles, appearing in a recent issue of *The Lancet*, have reported studies of dextran as a plasma substitute (10). The production and the molecular association of dextrans have been investigated (11).

Dextran is a glucose polymer which forms as a viscous gel during the growth of certain strains of *Leuconostoc* and other soil streptococci on a sucrose medium. The polymer is made up of α-glucopyranose units linked in the 1:6 position (3, 4, 6). "Degraded dextrans" have been described by Stacey and Youd (4*a*) and by Grönwall and Ingelman (2*a*, *c*). Stacey and Youd (4*a*, 4*b*) have eliminated nitrogen by heating dextran at 100° with 0.1 *N* HCl for fifteen minutes, and have found the relative viscosity of a 1% aqueous solution of the "degraded" product to be 1.01 as compared with a relative viscosity of 3.20 for 1% solutions of the unhydrolyzed dextran. Grönwall and Ingelman (2*b*) report a nitrogen-free preparation. They graph the viscosity of a number of "partially degraded" dextrans, and for intravenous testing (2*c*, page 10), they recommend a relative viscosity between 3.5 and 7 for 4% aqueous solutions of preparations; or in a more recent paper, Ingelman (2*d*) suggests a 6% solution but does not give a numerical viscosity range.

A large part of our work was carried out with a dextran which was supplied to us by the courtesy of Refined Syrups and Sugars, Inc.;[2] micro-Kjeldahl analyses showed only 0.03% to 0.04% of nitrogen. This gel had been stored under isopropyl alcohol (*p*H = approximately 6) for some months before we isolated the dehydrated white fibrous dextran. Dextran, which was obtained in our own laboratory[3] from cultures of *Leuconostoc mesenteroides*, contained 0.4% of nitrogen. The various dextran preparations were found to be soluble in ethylene diamine (7) and in acetamide as might be expected.

EXPERIMENTAL

Partial Purification of Crude Dextran.—Two-hundred-gram lots of the crude dextran were placed in cellophane sacks and dialyzed against distilled water until the dialysate gave only a slight positive test with Benedict's solution for reducing sugars. Between 1 and 2 equal weights of water diffused into the sack. Precipitation was carried out by adding the dextran solution slowly to a gallon of 95% alcohol with mechanical stirring. The thread formed best as the solution became about 85–90% of alcohol. The sample was finally dried at 110°. The yield was approximately 35% of the weight of the crude gel and had a specific rotation of +166°. After re-precipitation and re-drying the dextran: $[\alpha] = +181°$; moisture = 8–9%.

Autoclaving.—Autoclaving as a method of hydrolysis was carried out at 15 lb. pressure and a temperature of 250° Fahrenheit with the solutions in loosely-capped, screw-top bottles. The concentration of dextran, the concentration of acid, and the time intervals were varied.

The check on possible hydrolysis during sterilization of dilute neutral solutions was carried out with a 5% aqueous solution of partially degraded dextran. The relative viscosities of the 5% solution before and after hydrolysis were 4.59 and 4.56 centipoises, respectively.

Recovery.—In routine practice the solutions of "degraded" dextrans were filtered through Johns-Manville Celite to remove a flocculent sediment and reprecipitated by slow addition of the aqueous solution to alcohol to form an 80% alcoholic supernatant. The gum was redissolved, precipitated from a large volume of alcohol, and dehydrated with acetone. In several test runs the samples showed little change on subsequent extraction with 50% alcohol or after dialysis in a cellophane sack.

Viscosities and Fractionation.—Viscosities are reported in Tables I and II. For the values reported in Table I, the autoclaved solutions were filtered with suction to remove slight flocculent precipitates and aliquots of the hydrolyzates were diluted to volume to provide the 5% and 1% solutions tested. It is evident from viscosity measurements reported in Table I that the average molecular size is appreciably decreased by autoclaving even aqueous solutions at 15 lb. pressure for 4–5 hours. In the presence of dilute acid, the degree of partial degradation of dextran during convenient intervals of autoclaving appears to be fairly reproducible and to show an expected correlation with the hydrogen ion concentration.

For a better knowledge of the range of molecular weights in the "partially-degraded" fractions, it was suggested that a sample of each be distributed into three approximately equal fractions.[4] This procedure gave evidence of a wide range of molecular weights in the "degraded" preparations. Such a procedure could provide one possible means of repeated fractionation toward colloids of limited molecular-weight range. Actually a complication developed in attempts to fractionate with varying concentrations of aqueous alcohol. On the careful addition of alcohol to dilute aqueous solutions of dextran (2% to 6%), clouding occurs and subsequently two liquid phases form. For the degraded dextrans studied, the two phases appeared when the

TABLE I.—VISCOSITIES OF AUTOCLAVED SOLUTIONS OF DEXTRAN

Concentration	Time[a]	Viscosity 1%	Viscosity 5%
Original	...	3.72	..
10% dextran in H_2O	1 hr.	3.11	..
10% dextran in H_2O	4 hr.	2.95	..
10% dextran in 0.001 *N* HCl	15 min.	2.73	..
20% dextran in 0.001 *N* HCl	1 hr.	2.34	17.9
20% dextran in 0.0032 *N* HCl	1 hr.	1.48	4.9
20% dextran in 0.01 *N* HCl	15 min.	1.53	7.2
10% dextran in 0.01 *N* HCl	15 min.	1.28	6.6

[a] 15 lb. pressure at 250° F.

[2] We take pleasure in acknowledging our indebtedness to Refined Syrups and Sugars, Inc., of Yonkers, N. Y., for the generous gift of dextran.

[3] The bacteriological work was carried out by W. W. Carlson who had previously studied dextran (5).

[4] We are indebted to Dr. R. C. Briant for suggesting this method of studying the distribution and range of molecular weights of samples.

TABLE II.—FRACTIONATION IN AQUEOUS ALCOHOL (40% ALCOHOL)

	η_{sp}		$[\eta]^a$	
	5%	1%	5%	1%
Degraded V	19.0	2.0	0.52	0.694
Upper layer	4.0	1.38	0.28	0.38
Gelatinous lower layer	27.0	2.3	0.659	0.833
Degraded II	2.35	1.35	0.17	0.30
Upper layer	3.18	1.29	0.230	0.255
Gelatinous lower layer	4.63	1.50	0.307	0.40

[a] $[\eta] = \frac{2.303 \log \eta_{sp}}{\text{conc.}}$ (9).

aqueous-alcoholic solvent contained 35–45% of alcohol at 27°. Somogyi (8) has reported a similar solubility for glycogen.

Two samples of fractionation are presented in Table II. Dextran V had been prepared by autoclaving 35 Gm. of dextran with 175 cc. of 0.04 *N* hydrochloric acid (i.e., a 20% dextran solution) for 50 minutes at 15 lb. pressure and 250° F. Thirty-one grams of degraded dextran were recovered after precipitating three times and had a relative viscosity of 2.0 for a 1% aqueous solution. Fractionation in 40% alcohol gave a recovery of 66% of the degraded dextran in the lower, more viscous layer. The other sample in Table II was obtained by hydrolyzing dextran with 0.1 *N* hydrochloric acid as described by Stacey and Youd (4*a*) and shows much less spread in the average molecular weight of the fractions. It is noticeable that the apparent intrinsic viscosity of these preparations increases with dilution.

Animal Testing.—The possible effects of various samples of degraded dextran were tested by intravenous injection into mice and rabbits by Drs. M. L. Menten and A. M. Carpenter of the Children's Hospital of Pittsburgh. The kidneys of these animals showed only minor microscopic changes. There was, however, evidence that the dextran was being taken out of circulation by liver and spleen. Because of the reaction in these two organs it was not deemed advisable to continue the work with a view to using dextran on patients with nephrosis.

SUMMARY

1. Partial hydrolysis of dextran has been studied by autoclaving solutions of various concentrations in the presence of water or dilute hydrochloric acid. Viscosities of hydrolyzates are reported.

2. Relative viscosities are reported for some recovered partially hydrolyzed dextrans.

3. Sterilization by autoclaving for thirty minutes caused no measurable change in the relative viscosities of 5% solutions of partially hydrolyzed dextrans.

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A Quantitative Assay Method for Pyrogens*

By WALTHER H. OTT

A quantitative assay is described for the determination of pyrogens in water and in biological products. Evaluation is based upon a standard curve, the derivation and application of which are explained. Individual rabbits were found to differ widely and characteristically in their response to a standard pyrogen. Animals used in the experiments reported were chosen so that variability was minimized.

IN A RECENT study of factors influencing the evaluation of pyrogen tests (1), data were presented indicating a direct relationship between the logarithm of the dose and the average maximum temperature rise following intravenous injection into rabbits. Reasonably satisfactory linearity was observed over a 500-fold range in dosage. This finding has now been employed in developing a quantitative assay method for pyrogens in water and biological products.

The method of evaluation employing a standard dosage-response curve as described in the following assay procedure is a new method for pyrogen tests and represents a distinct improvement in bioassay technique over the essentially

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"all-or-none" procedure currently used by many laboratories. The fixed standard curve is satisfactory from the biometrical standpoint as long as experimental error is not small enough to indicate significant differences between individual assays.

ASSAY PROCEDURE

All the tests were conducted under conditions of routine procedure employing the techniques reported from this laboratory (1, 2). Male albino rabbits received from various sources and weighing from 2 to 4 Kg. were used. No attempt was made to restrict normal growth of the animals. Instead, they were given free access to an adequate laboratory diet except while on test. Each animal was given a rest period of at least three days between tests.

The animals were restrained for the duration of the test in specially constructed boxes. Rectal temperatures were determined to the nearest 0.1° C. by the use of stationary rectal thermocouples. Iron and constantan wires of No. 24 B & S gauge (Leeds and Northrup Co., Inc.) were used for the thermocouples and leads which were connected directly to selector switches having low thermal potentials. Soft plastic tubing having an outside diameter of 3 mm. was employed as conduit for the wires.

Each rabbit was subjected to a training period of several weeks (two tests per week) before being used in the quantitative assays. The training period varied in length with individual rabbits and was necessary for valid responses in the initial tests with new animals. For the first week or two the animals were merely held in the boxes for the duration of a test to become accustomed to the restraining procedure. Then they were given two mock tests (no injections) per week until their control temperatures fell within the acceptable range of 37.0° to 38.9° C. for two successive tests. This temperature range, while lower than that specified in the U. S. P. XIII pyrogen assay, appears to be the normal for animals handled and treated according to this assay procedure.

Control temperatures were read after the rabbits had been held in the boxes for one and one-half to two hours and were followed immediately by the injections. Three subsequent temperatures were read at hourly intervals. The maximum temperature rise following the injection was calculated as the difference between the control temperature and the highest of the three subsequent hourly temperatures. The maximum rise was recorded as zero whenever none of the subsequent temperatures was higher than the control temperature.

In assaying water and biological materials, each sample was tested on three animals, the same dose per Kg. being given each rabbit. When greater accuracy was desired in the result, 4, and occasionally 5, animals were used per sample. Four rabbits per sample appeared to be most efficient on the basis of animals and effort required and precision obtainable. The average of the maximum rises thus obtained was used in determining the equivalent pyrogenicity by reference to a table of values (Table I) calculated from the standard curve.

For the purpose of the quantitative assay, a "Pyrogen Unit" was established as the pyrogenicity present in 0.1 mg. of a dry pyrogen standard ("PS1").[1] This material was prepared by saturating with sodium chloride a pyrogenic vaccine (a Pseudomonas) which was then precipitated with ethanol and dried with ether. The above unitage was chosen because under the conditions of the test the intravenous injection of 0.1 mg. of this material per Kg. (one pyrogen unit) produced an average thermogenic response of 1.0° C. per rabbit.

TABLE I[a]

Maximum Temp. Rise, Av. ° C.	Pyrogen Units in Dose/Kg.,	Maximum Temp. Rise Av. ° C.	Pyrogen Units in Dose/Kg.
0.0	0.05 or less	1.3	2.4
0.1	0.07	1.4	3.3
0.2	0.09	1.5	4.4
0.3	0.13	1.6	5.9
0.4	0.17	1.7	7.9
0.5	0.23	1.8	11
0.6	0.31	1.9	14
0.7	0.41	2.0	19
0.8	0.55	2.1	26
0.9	0.74	2.2	35
1.0	1.00	2.3	46
1.1	1.3	2.4	62
1.2	1.8	2.5	84

[a] Values for "Pyrogen Units in the dose per Kg." corresponding to "average maximum temperature rise in ° C." as calculated according to the standard curve: "average maximum temperature rise in ° C. = 1.00 + 0.78 (log pyrogen units in the dose per Kg.)".

In order to insure continuously uniform sensitivity of an average response of 1.0° C. to one pyrogen unit per Kg., the rabbits in the assay colony were periodically tested with the pyrogen standard. Animals that repeatedly gave responses of less than 0.6° C. or more than 1.7° C. to one pyrogen unit were removed from the colony.

The linear equation for the standard dosage-response curve as experimentally determined in this laboratory was $Y = 1.00 + 0.78X$. In this equation Y is the average maximum rise in ° C.; $1.00 = a$, which is the average maximum rise for one pyrogen unit per Kg. (that is, when $X = 0$); $0.78 = b$, which is the slope of the curve (that is, an increase of 0.78° C. in maximum rise per tenfold increase in dose of pyrogen); and X is the logarithm of the dose per Kg. in pyrogen units.

By this method of evaluation the number of pyrogen units is estimated directly for a given quantity of substance (i.e., the dose administered). Until the pyrogen, or pyrogens, is chemically defined and potency can be stated in terms of weight of a pure compound, the pyrogen unit as defined above should suffice as a measure of pyrogenic activity.

EXPERIMENTAL

The position ($a = 1.00$) and slope ($b = 0.78$) for the fixed standard curve were adopted as the result of a series of multi-dose experimental assays designed to furnish information on precision and variability of the testing procedure. For these assays, animals were selected at random from the test animals in use at the time. Two or more graded doses

[1] The author is indebted to Dr. D. M. Tennent for preparation of the pyrogenic powder used in these studies.

of the pyrogen standard were used in each assay and equal numbers of animals were given each of the doses in an assay. Each assay was performed on a different day. Biometrical analyses of the data were conducted according to methods described by Snedecor (3) and Bliss (4).

Results of these experimental assays (Table II) showed that both the sensitivity to pyrogen and the slope of the dosage-response curve were apparently maximum and relatively constant in rabbits averaging over 2 Kg. in weight and under 39° C. in control temperature (Expts. 2 and 3). In younger, smaller rabbits (Expt. 1) both sensitivity and slope were appreciably less although all animals had been presumably well trained prior to the tests. Nevertheless, variability tended to be lower in the heavier animals, which in addition had been used in routine assays for a longer time.

The pooled slope of 0.78 calculated for Expts. 2, 3, and 4 was therefore considered the most reliable estimate available for the slope of the curve with animals weighing over 2 Kg., having control temperatures below 39° C., and showing sensitivities of approximately 1° C. to one pyrogen unit per Kg. It was interesting to note that an analysis of covariance of the data presented by Robinson and Flusser (5) showed the slope of their dosage-response curve to be 0.79 (mean square for linear regression = 25; $F = 173$ *HS*). The fact that linear regression has been very highly significant whenever tested ($F = 15$ to 98 in Table II) is further proof of the existence of the dosage-response relationship in the pyrogen assay.

An inverse relation between body weight and control temperature was indicated in the experiments cited in Table II. This relationship was found to be

TABLE II.—SUMMARY OF EXPERIMENTAL ASSAYS WITH A STANDARD PYROGEN ("PS1")

Experiment		1	2	3	4	5
Mean Body Wt., Gm.		1690	2250	3110	2970	3210
Mean Control Temp., ° C.		39.15	38.78	38.60	38.06	38.40
Source of Variation[a]						
Between assays	*df*	7	1	2	2	2
	M.S.	0.426	0.547	0.032	0.262	0.138
	F	2.1 *NS*	2.8 *NS*	0.3 *NS*	2.6 *NS*	1.1 *NS*
Linear regression	*df*	1	1	1	1	1
	M.S.	11.2	12.8	10.8	7.59	1.93
	F	56 *HS*	64 *HS*	98 *HS*	77 *HS*	15 *HS*
Error	*df*	59	63	32	20	20
	M.S.	0.200	0.198	0.110	0.099	0.120
	F	1	1	1	1	1
Sensitivity, a[b]		0.80	1.11	1.04	1.20	0.57
Slope, b		0.60	0.77	0.78	0.80	0.40
Variability, s		0.447	0.445	0.332	0.315	0.347
Precision, s/b		0.75	0.58	0.42	0.39	0.86

[a] As determined in the analysis of covariance between log dose per Kg. and ° C. max. temp. response; *df* = degrees of freedom, *M.S.* = mean square, *F* = ratio between indicated variance and error variance, *HS* = highly significant source of variation ($P < 0.01$), *NS* = not significant.

[b] a and b in linear regression equation "average maximum temperature rise = $a + b$ (log pyrogen units per Kg.)." a is the average maximum response to one pyrogen unit per Kg., and b is the slope of the dosage-response curve. s = standard deviation, the square root of the error mean square in the analysis of variance.

The value s/b is one standard deviation in terms of log dose. Low values indicate high precision.

Neither differences in mean responses to pyrogen ("Between assays") nor in individual slopes ($F = 1.0$ or less, not tabulated) were significant. Consequently, use of the fixed standard dosage-response curve was valid because individual assay curves did not differ significantly among themselves. Should situations occur in which position or slope of the response curve or both differ significantly from one assay to another, it would be preferable to determine, and apply, the position and slope of the curve for each assay.

In a group of rabbits selected especially for high sensitivity, the sensitivity in the experimental assays (Expt. 4) was slightly above that in Expts. 2 and 3 while the slope was essentially unchanged. In a second group of rabbits, selected for low sensitivity, sensitivity and slope were so low (Expt. 5) that such animals were regarded as unsuitable for use in acceptable quantitative assays. Because the above results indicated a tendency toward higher sensitivity in animals having low control temperatures and high body weights, the minimum weight used for routine assays was increased to 2 Kg. The maximum weight has been approximately 4 Kg. largely as a matter of convenience.

very marked in the assay colony when animals weighing 1.3 to 2.9 Kg. and averaging 2.10 Kg. were used (1), as shown in Fig. 1. In the higher weight range of 2 to 4 Kg. this relation apparently ceased to exist. During a twelve-month period in which over 96% of the animals weighed between 2 and 4 Kg., the average control temperature remained uniformly close to 38.5° C. throughout this weight range.

The heavier animals in most instances had been subjected to the routine procedure for several months longer than the lighter animals, and consequently were expected to be more accustomed to the test. However, recent experience indicates that previously unused rabbits of 2 to 3 Kg. show the typically low control temperatures after relatively short training periods. Apparently the relation between control temperature and body weight (and age) is not primarily a function of training. The high weight range has resulted in increased uniformity of the control temperatures, which probably accounts for some of the decrease in variability of responses to pyrogen (Table II).

By means of the periodic tests on each animal in the assay colony with one pyrogen unit per Kg. for

the purpose of maintaining uniform responses to pyrogen, it was found that individual rabbits in general appeared to have characteristic sensitivities to pyrogen. Maximum temperature rises of the 169 rabbits in the initial test ranged from 0.0 to 2.8° C. with a mean of 1.13° C. and a standard deviation of 0.47° C. A retest with one pyrogen unit per Kg. on the 15 animals giving rises of 1.8° C. or above and on the 12 rabbits giving rises of 0.5° C. or less in the initial test showed that responses in the retest closely paralleled the responses in the first test (Table III). How long a characteristic sensitivity to pyrogen is maintained by the individual rabbit has not yet been determined. Although the rabbits with high sensitivity might be desirable from that standpoint, the proportion has been too small to be of value at present (5 to 9%).

The periodic tests with one pyrogen unit per Kg. have also shown that the distribution of the individual maximum temperature rises conforms closely to the normal distribution (Table IV). In the first test, referred to above, the distribution was slightly asymmetrical (significant g_1, $P = 0.02$). However, in the next two tests, eight and eleven months later, the distributions did not deviate significantly from normal. Under such conditions the mean of a group of maximum rises is expected to provide an unbiased estimate of the pyrogenic response. The mean is therefore more reliable than any individual maximum rise.

TABLE III.—COMPARISON OF RESPONSES IN TWO GROUPS OF RABBITS SELECTED ON THE BASIS OF SENSITIVITY TO PYROGEN. THE DOSE IN EACH TEST WAS ONE PYROGEN UNIT PER KG.

Relative Sensitivity	No. Rabbits	Test	Maximum Temperature Rise Mean, ° C.	Maximum Temperature Rise Range, ° C.	Mean Control Temp., ° C.	Mean Body Weight, Kg.
High	15	1	2.02	1.8–2.8	38.12	2750
		2	1.70	1.0–2.3	38.25	2820
Low	12	1	0.33	0.0–0.5	38.72	2900
		2	0.74	0.5–1.3	38.58	3000

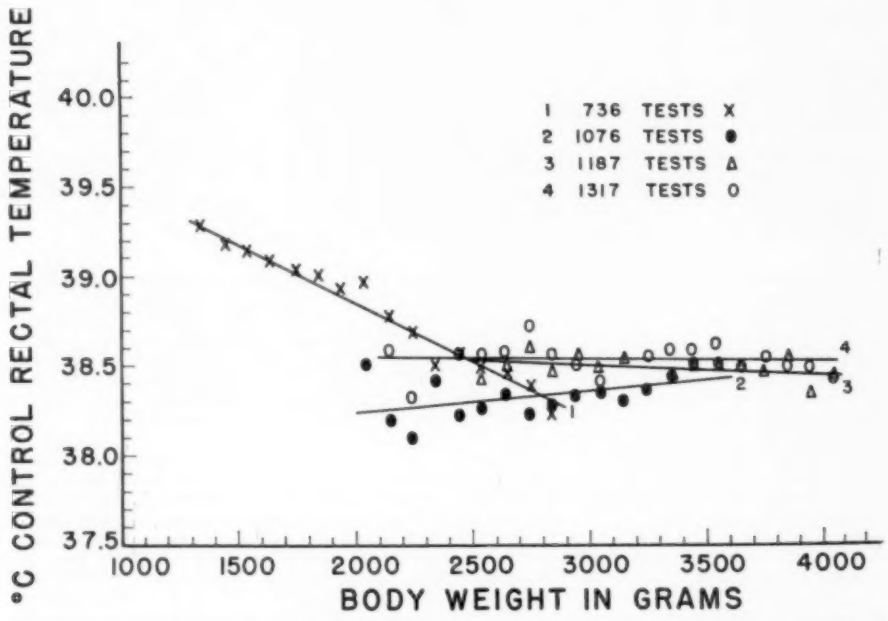

Fig. 1.—Curves showing the relation between control temperature and body weight for different weight ranges of rabbits restrained for one and one-half to two hours in special boxes for the pyrogen assay. Each point represents the average control temperature of 12 to 152 animals whose weights fell within the corresponding 100-Gm. interval during the course of one month's routine tests.

Curve I represents data obtained one month prior to institution of the quantitative assay. Curves II, III, and IV show the constancy of average control temperatures of rabbits throughout the higher weight range. These curves represent data collected during one month of routine assays 4, 8, ond 15 months, respectively, subsequent to the period represented by Curve I.

TABLE IV.—STATISTICS ON MAXIMUM TEMPERATURE RISES FOLLOWING INJECTION OF ONE PYROGEN UNIT PER KG. TO ALL RABBITS IN THE ASSAY COLONY

Test No.	No. Rabbits	Mean Rise, ° C.	Standard Deviation, ° C.	Deviations from Normality[a]			
				$g_1 \pm S.E.$	P	$g_2 \pm S.E.$	P
1	169	1.13	±0.47	0.44 ± 0.19	0.02	0.62 ± 0.37	0.10
2	165	1.09	±0.42	−0.17 ± 0.19	0.37	0.03 ± 0.38	0.99
3	150	1.09	±0.39	0.24 ± 0.20	0.22	0.11 ± 0.39	0.80

[a] Calculated by procedures described by Snedecor (3).

In the previous study (1) it was found that the maximum rise was inversely proportional to the control temperature in addition to being a function of the dose of pyrogen. Some progress has been made in reducing the effect of control temperature so that the maximum rise is more closely related to the amount of pyrogen administered. In the initial experiment on 169 rabbits, correlation ($r = -0.345$) and linear regression ($b = -0.338$) between maximum temperature rise and control temperature were highly significant ($P < 0.01$). Omission of the data on the 27 rabbits of high and low sensitivities, thereby leaving a range of 0.6 to 1.7° C. in response to one pyrogen unit, reduced very significantly the correlation ($r = -0.234$) and regression ($b = -0.169$). These statistics, however, were still significant ($P = 0.01$), indicating that a greater reduction in range would have been necessary to reduce the relation to nonsignificance.

Values obtained in the second periodic test (165 rabbits) were almost identical before and after omission of the data on 28 rabbits of high and low sensitivities. However, in the third test (150 animals) the correlation ($r = 0.026$) and regression ($b = 0.025$) were almost zero and were unchanged by omission of data on 17 rabbits of high and low sensitivities. It appears that periodic elimination of rabbits giving responses above 1.7° C. and below 0.6° C. to one pyrogen unit per Kg. has resulted in an assay colony in which the maximum temperature rise is not significantly affected by the corresponding control temperature under the conditions of these tests.

DISCUSSION

The precision of this pyrogen assay is relatively low compared to other biological assays (6). However, a direct means for increasing the precision of the assay value on a given sample is to increase the number of animals used. The standard error of the pyrogenicity is inversely proportional to the square root of the number of animals used in the assay, as shown in the following formula for % *S.E.* (4):

$$\%\ S.E. = \frac{s \times 230.3}{b \times \sqrt{n}}$$

Thus, the % *S.E.* could be reduced one-half by an increase of fourfold in the number of rabbits per assay. Of course, any reduction in variability (s) whenever practical, or increase in slope (b), if possible, would also tend to increase the precision.

The data in Tables II and IV indicate that the average standard deviation (s) for the individual response of rabbits under our testing conditions probably has been close to ±0.40° C. From this value the standard error of the pyrogenicity determined from the average response by a group of 4 rabbits, for example, was calculated by means of the above formula as approximately ±60% of the estimated pyrogen unitage, i.e.,

$$\%\ S.E. = \pm\frac{0.40 \times 230.3}{0.78 \times \sqrt{4}}.$$

In a series of samples varying from 0.1 to 1.0 pyrogen units per dose per Kg., and assayed from 2 to 11 times each with 4 rabbits per assay, the average standard error of an individual assay value was calculated as ±55% (error standard deviation × 230.3). Actual reproducibility thus agreed closely with that expected theoretically on the basis of inherent precision of the assay.

As is customary in adopting new methods of evaluation, each laboratory should determine the sensitivity to a standard pyrogen and the slope of the dosage-response curve under their testing conditions. The mean sensitivity thus determined should be substituted for a in the equation, $Y = a + bX$, and the observed slope used in place of b. A table of values for pyrogen units corresponding to mean responses can then be calculated similar to Table I presented above.

The experimental determination of sensitivity and slope is not difficult, as study of appropriate biometrical references will show (e.g., 4). The following example is given to illustrate an experimental assay for this purpose:

Dose, Description	Low (L)	High (H)
Dose, Pyrogen Units/Kg.	0.2	5.0
Dose, in logs	−0.699	0.699
Response, individual maximum rises in °C.	0.4	1.5
	0.7	1.4
	0.5	1.8
	0.5	1.6
Response, total	2.1	6.3

Orthogonal coefficients (apply directly to total responses),

for sensitivity	$v = +1$	$+1 = 8.4$
for slope	$w = -1$	$+1 = 4.2$

Mean sensitivity to one pyrogen unit per kg. $= a = \frac{v}{N_L + N_H} = \frac{8.4}{8} = 1.05$° C

Note that mean log dose is 0, corresponding to one pyrogen unit. N = number of responses to dose indicated.

$$\text{Slope} = b = \frac{w}{iN} = \frac{4.2}{1.398 \times 4} = 0.75.$$

i = log dose interval between L and H.

Corresponding equation therefore is:

Av. Max. Rise = 1.05 + 0.75 (log pyrogen units per Kg.).

For a reliable determination of sensitivity and slope, a series of such assays should be conducted on animals chosen in the same manner as for the routine tests, and the responses totaled for the calculations. Preliminary tests might be necessary to determine

whether or not the above doses provoke the expected responses. At any rate the dose interval in a two-dose assay should be at least tenfold for satisfactory evaluation of the response to pyrogen. The doses of 0.2 and 5.0 pyrogen units per Kg. were chosen because of ease in periodically checking directly the sensitivity and slope of the standard curve, as indicated in the example.

SUMMARY

1. A quantitative assay has been developed for the determination of pyrogens in water and biological products. Evaluation is based upon a standard curve having the equation "average maximum temperature rise in °C. = 1.00 + 0.78 (log pyrogen units per Kg.)."

2. A "pyrogen unit" was arbitrarily established as the amount of pyrogen in 0.10 mg. of a standard preparation. Under conditions of this test one pyrogen unit caused an average maximum rise of 1.0° C. in rectal temperature.

3. The standard error of an assay value based on the average response of 4 rabbits was estimated at approximately ±60 per cent based on observed reproducibility as well as on inherent precision of the assay.

4. Tabular values were given for evaluation of assays conducted under conditions conforming to the statistics of the standard curve, and a procedure was illustrated for determination of sensitivity and slope of the dosage-response curve.

5. Individual rabbits appeared to differ widely and characteristically in their response to a standard pyrogen. Use was made of this fact in choosing animals so that variability of responses was minimized.

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The Preparation and Composition of Groundnut Cake, *Arachis Hypogea* (Peanut) Hydrolysate*,†,‡

By R. RAGHUNANDANA RAO and P. R. VENKATARAMAN

The preparation of peanut cake hydrolysate by use of papain is described. The composition of the hydrolytic products was determined and the results are reported.

IT HAS been widely accepted that the peanut is a fairly complete food from the standpoint of its chemical composition and ready digestibility (1). It contains an abundance of energy-producing constituents in the form of fat and carbohydrate, several vitamins of the B group (7–10), and is also a very rich source of vitamin E. The work of Johns and Jones (2–4), Brown (5), and Rose (6) have shown that arachin and conarachin, the proteins occurring in the peanut, contain significant proportions of indispensable amino-acids.

In India, peanut cake, commonly known as groundnut cake, is used largely either as fertilizer or fodder. In the production of antibiotics by microorganisms (11, 12), papain hydrolysate of groundnut cake as a medium has been used with very satisfactory results comparable with those of synthetic media. In this communication the preparation and composition of the hydrolysate are described.

EXPERIMENTAL

Preparation of Materials and Proximate Composition of the Groundnut Cake.—The peanut cake was supplied by Sri RamaKrishna Oil Mills, Bangalore, India. The cake was powdered and passed through a 60-mesh sieve. The papain used in these experiments was prepared by treating fresh latex of unripe papaya with ten volumes of dry acetone, filtering through a Buchner funnel, and drying in vacuum. In Table I the mean proximate chemical composition of the cake expressed as percentage of the dry material is presented.

Preparation and Composition of Groundnut Cake Hydrolysate.—Fifty grams of the finely powdered peanut cake was dispersed in 300 cc. of water in a

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TABLE I.—PROXIMATE COMPOSITION OF GROUNDNUT CAKE

Determinations	Dry Material, %
Moisture[a]	5.01
Protein[c]	51.88
Ether extractives[a]	6.82
Carbohydrate[b]	26.63
Fiber[a]	4.16
Ash[a]	5.50

[a] Methods of the Association of Official Agricultural Chemists (13).
[b] Hassid, McCready and Rosenfals (14).
[c] Micro-Kjeldahl method using the digestion mixture of Chibnall, Rees, and Williams (15).

500-cc. round-bottom flask fitted with a condenser. One gram of papain was added to the mixture which was then shaken vigorously for ten minutes. The mixture was heated in a water bath maintained at a temperature of 73–77° for five hours and was shaken frequently. At the end of the period, 20 cc. of 0.5 *N* sulfuric acid was added and the mixture was boiled for fifteen minutes under reflux and then filtered hot or centrifuged. The residue was washed twice with 75- and 50-cc. portions of hot water. The combined filtrates or centrifugates and washings were clarified as necessary, by refiltration through filter paper and were made up to a volume of 400 cc. with water containing 1 cc. of chloroform.

The hydrolysate gave positive tests with the Biuret, the Millon, and the Hopkins-Cole reagents. Cystine was present in the hydrolysate in very small quantities as indicated by its faint reaction with uric acid reagent. The mean *p*H of the hydrolysate was 5.3 and gave only a faint turbidity with 5% trichloracetic acid. Under the conditions of hydrolysis, 12.5% of the carbohydrate and 66.3% of nitrogen of the groundnut cake solubilized. The composition of the hydrolysate is summarized in Tables II and III. Recovery of added tyrosine and tryptophane to the hydrolysate was good.

TABLE II.—HYDROLYSATE COMPOSITION

Determinations	Gm. per Liter of Hydrolysate			
	Expt. No. I	Expt. No. II	Expt. No. III	Expt. No. IV
Total solids[a]	57.70	52.20	57.60	55.50
Total nitrogen[b]	6.85	6.80	6.89	6.66
Amino nitrogen[c]	1.49	1.52	1.39	1.55
Amide nitrogen[d]	0.73	0.70	0.77	0.73
Phosphotungstic acid precipitable nitrogen[e]	4.25	4.12	4.38	4.33
Tryptophane[f]	0.51	0.53	0.51	0.49
Tyrosine[f]	2.18	2.04	1.86	2.03
Starch[g]	4.14	...	4.18	...
Ash[h]	2.98	...	3.02	...

[a] 10-cc. aliquot of the hydrolysate was dried at 100° to constant weight.
[b] Micro-Kjeldahl method.
[c] Sorensen formol titration method.
[d] Amide nitrogen was determined in 2 cc. aliquots of the hydrolysate according to Damodaran (16).
[e] Basic nitrogen was determined on the phosphotungstic acid precipitate after decomposition with barium hydroxide solution (17).
[f] Tryptophane and tyrosine were determined by the method of Folin and Ciocalteu (18) after hydrolyzing 2-cc. aliquots with 2 cc. 40% sodium hydroxide in a steam bath for eight hours.
[g] Method of Hassid, McCready, and Rosenfals (14) on 25-cc. aliquots.
[h] Dry material obtained from 20-cc. aliquots of the hydrolysate was ashed at 600°.

TABLE III.—NITROGEN DETERMINATION

Form of Nitrogen	Mean Value Expressed as % Total Nitrogen
Amino nitrogen	21.09
Amide nitrogen	10.76
Phosphotungstic acid precipitable nitrogen	62.79
Tyrosine	2.23
Tryptophane	1.03
Mean complexity of peptone = 4.75	

From Table II it is evident that the ash constitutes 0.3% of the hydrolysate. The ash gave positive tests for phosphorus, potassium, iron, calcium, magnesium, and aluminum. It contained on an average of three analyses 21.35% potassium (19) 0.17% iron (20), and 3.9% phosphorus (21).

SUMMARY

1. The preparation of the groundnut cake (peanut, *Arachis hypogea*) hydrolysate with papain and its composition are described.

2. Nearly 66 per cent of the total nitrogen of the groundnut (peanut) cake could be dissolved under the conditions of the experiment.

3. The hydrolysate contains a peptone of a mean complexity of 4.57. Of the total nitrogen of the hydrolysate 63 per cent is precipitable by phosphotungstic acid. Tryptophane and tyrosine are present in significant amounts.

4. Ash constitutes 0.3 per cent of the hydrolysate and has a very high potassium content with appreciable quantities of iron and phosphorus.

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Influence of Amphetamine Sulfate on Cerebral Metabolism and Blood Flow in Man*,†

By BENEDICT E. ABREU, GRANT W. LIDDLE, ARTHUR L. BURKS, VIOLETTE SUTHERLAND, HENRY W. ELLIOTT, ALEXANDER SIMON and LESTER MARGOLIS‡

Cerebral blood flow, cerebral oxygen and glucose utilization and mean blood pressure were determined in a series of humans before and after the intravenous administration of amphetamine. Amphetamine sulfate, 20 mg., increased mean blood pressure, and reduced cerebral blood flow and oxygen uptake in the majority of subjects.

THE OBJECTIVE and subjective manifestations of central nervous system stimulation produced by ampetamine sulfate in most mammals are well recognized and adequately documented in the pharmacologic and medical literature. Numerous reports have indicated the usefulness and mechanism of action of ampetamine in stimulating the depressed mammalian central nervous system. The effects on the metabolism of brain slices have been described by Mann and Quastel, (1) but an adequate investigation of cerebral metabolism in the intact mammal has been delayed because of inadequate procedures for determining cerebral blood flow. Dumke and Schmidt (2), by means of the bubble flow meter, have shown that amphetamine decreases cerebral blood flow in the anesthetized monkey, and Schmidt, Kety, and Pennes (3) have found that it decreased cerebral metabolism and blood flow in 2 anesthetized monkeys and increased it in a third. The introduction of the Kety-Schmidt nitrous oxide technique (4) has made possible the ready investigation of the effects of drugs upon the cerebral vascular bed and upon the over-all metabolism of the intact mammalian brain. It has been emphasized by Schmidt (5) that only three easily available mammals possess a cerebral vascular circuit with a minimum of anatomic peculiarities allowing for the use of such methods. These are the rabbit, monkey, and man. Since this procedure can be readily employed in man without general anesthesia as opposed to its necessity in the rabbit or monkey, a number of investigations are now in progress attempting to elucidate the action of various drugs upon cerebral blood flow and metabolism in man. The present study is concerned with the investigation of the influence of amphetamine sulfate upon cerebral blood flow and oxygen and glucose uptake in human subjects.

EXPERIMENTAL

Nine adults of different sexes with ages varying from twenty to forty years volunteered as subjects. They were all individuals in good physical health but were voluntary patients in the Langley Porter Clinic with a number of psychiatric complaints. The Kety-Schmidt nitrous oxide technique (4) or a modification devised in this laboratory (6) was employed for the estimation of cerebral blood flow. Mean blood pressure was measured by means of a damped mercury manometer which was connected to the arterial blood sampling manifold. Blood samples were obtained from the femoral artery and one internal jugular vein by means of hypodermic needles (3 in., 19 Ga.) connected by fine bore plastic tubing to separate manifolds consisting of four 3-way metal stopcocks to which were connected sampling syringes. The system was sterilized by autoclaving prior to use and was filled with sterile heparin solution (1%) immediately prior to arterial and venipuncture. The syringes were all lubricated and sealed with a fine coating of liquid petrolatum and were also heparinized.

Oxygen and nitrous oxide were determined in the blood samples by means of the Van Slyke-Neill method (7) for the determination of oxygen and by the Kety modification (8) of the Orcutt-Waters procedure for nitrous oxide. Glucose was determined in an aliquot of the same blood samples by the Somogyi-Folin method (9, 10). Glucose determinations were done in only four experiments.

A control determination of cerebral blood flow was obtained after which the subject was allowed to breathe room air for 20 minutes. During the last 5 minutes of this period amphetamine sulfate 20 mg., made to 10 cc. volume in sterile distilled water, was administered intravenously. Cerebral blood flow and metabolism were then determined during the next five to ten minutes when the mean blood pressure appeared to be stabilized.

RESULTS AND DISCUSSION

Amphetamine sulfate, 20 mg., intravenously, raised the mean blood pressure in all of the subjects but did not consistently alter cerebral blood flow, cerebral oxygen or glucose uptake. The

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results obtained in each subject are shown in Fig. 1 expressed as a percentage change from the control. Actual values for mean blood pressure, cerebral blood flow and cerebral oxygen and glucose uptake are given in Table I.

The smallest elevation of mean blood pressure was 17 mm. Hg and the greatest 60 mm. Hg. The range of variance in per cent from the control was from −40% to +14% in cerebral blood flow; from −37% to +46% in oxygen uptake; and from −38% to +33% in glucose uptake. It is of particular interest that changes in oxygen uptake, although not always of the same magnitude, were in the same direction as those in blood flow. Changes in glucose uptake could not be correlated with either. (See Fig. 1.) In six of

TABLE I.—INFLUENCE OF AMPHETAMINE SULFATE, 20 MG., I. V., ON MEAN BLOOD PRESSURE, CEREBRAL BLOOD FLOW AND OXYGEN AND GLUCOSE UPTAKE IN MAN

Subject[a]	Mean Blood Pressure, Mm. Hg		Cerebral Blood Flow, Cc./100 Gm./Min.		Cerebral Oxygen Uptake, Cc./100 Gm./Min.		Glucose Uptake, Mg./100 Gm./Min.	
	Before Rx[a]	After Rx[b]	Before Rx	After Rx	Before Rx	After Rx	Before Rx	After Rx
1	70	95	81	52	5.6	3.5	14.0	8.65
2	85	125	70	54	4.3	3.6	...	...
3	75	110	41	51	2.6	3.8	4.7	4.85
4	90	130	78	83	3.7	4.8	6.2	8.3
5	80	115	55	43	4.0	3.1	14.5	16.8
6	93	115	50	30	4.5	3.2	...	...
7	100	120	36	31	2.7	2.3	...	...
8	100	108	70	58	4.1	3.5	...	...
9	95	102	35	40	2.7	3.0	...	...

[a] Control mean blood pressure.
[b] Mean blood pressure at end of cerebral blood flow determination following Rx.

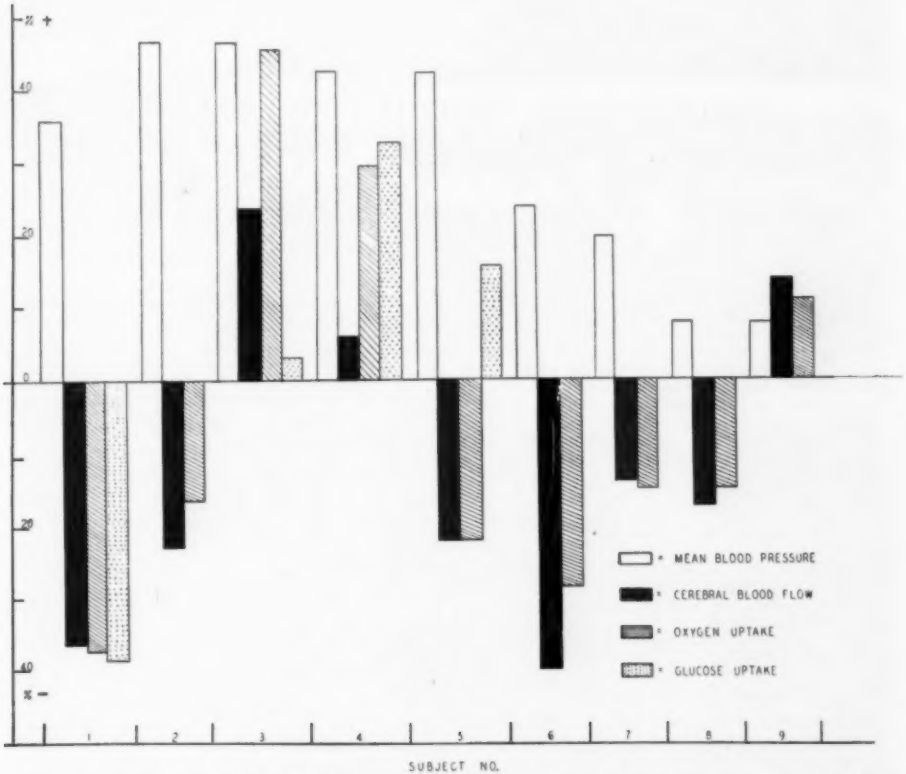

Fig. 1.—Influence of amphetamine sulfate, 20 mg., intravenously on mean blood pressure, cerebral blood flow, cerebral oxygen and glucose uptake in man (expressed in per cent from the control).

the nine subjects both cerebral blood flow and oxygen uptake were reduced. Two of the three other subjects (Nos. 3 and 4) showed a marked rise in oxygen uptake.

A correlation of these observations with the psychiatric findings and diagnosis will be published elsewhere.

From these observations it is difficult to arrive at a decision as to the effects on cerebral blood flow, cerebral oxygen and glucose uptake which should be predictable from what is otherwise known of the pharmacology of amphetamine. However, it is expected that as experience is broadened with the use of this agent in various psychic states and in "psychically normal" individuals a base line for prediction of these effects will be available.

SUMMARY

Amphetamine sulfate, 20 mg., intravenously, increased the mean blood pressure of 9 adult humans by values ranging from 17 mm. Hg to 60 mm. Hg. In 6 of the 9 subjects both cerebral blood flow and oxygen uptake were reduced. In 2 of the 3 remaining subjects cerebral oxygen uptake was definitely elevated, while in the third there was only a slight increase above this individual's control level. Glucose uptake was reduced in one, unchanged in one, and elevated in two subjects.

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Some Effects of Meperidine (Demerol) on Gastro-Enteric, Extra-Hepatic Biliary, and Cardiovascular Activity*,†

By RAYMOND W. PICKERING, BENEDICT E. ABREU, DAVID F. BOHR and WILLIAM F. REYNOLDS‡

A reinvestigation of some of the pharmacologic properties of meperidine indicates that atropine-like action cannot be demonstrated on the small intestine, extra-hepatic biliary tract or cardiovascular system of the dog. A decrease in gastric emptying in man cannot be interpreted as antispasmodic action. Evidence is cited which would place meperidine in a pharmacologic class with morphine rather than atropine with respect to its effect on structures innervated by the autonomic nervous system.

THE gastro-enteric effects of the analgetics meperidine (Demerol) and Methadone have recently been investigated by Scott and his co-workers (1) who have shown that both of these agents increase the motility of the small bowel. Gruber, Hart, and Gruber (2) observed similar effects on the duodenum and ileum of dogs. Yonkman, Noth, and Hecht (3) found that meperidine stimulated the ileum of dogs. However, it is still commonly stated that meperidine is a smooth muscle depressant and that it resembles atropine in many of its effects on other systems (3–4). In view of these apparent contradictory statements which have appeared in the literature, it was thought that a reinvestigation of some of the pharmacologic properties of meperidine on certain structures innervated by the autonomic nervous system was indicated.

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‡ We are indebted to Dr. L. L. Stanley, Chief Prison Medical Officer, California State Prison, San Quentin, Calif., who made the facilities of the prison hospital available to us. We also wish to thank Mr. W. Newcomb for his assistance in obtaining the X-ray photographs and the people who volunteered for this experiment.

EXPERIMENTAL

Animal Studies

A. Intestinal Activity.—The activity of the small intestine was investigated in 9 dogs anesthetized with sodium pentobarbital, 25–30 mg./Kg., intra-

peritoneally. Single balloons were placed in the jejunum and ileum through an abdominal incision and contractions were recorded on smoked kymograph paper by means of an air-water transmission system connected to sensitive Marey tambours.

B. Extra-Hepatic Biliary Activity.—A chronic preparation was utilized in which a mushroom catheter was placed in the gall bladder of a dog. The animal was trained to lie quietly without anesthesia and the activity of the gall bladder and magnitude of biliary secretion were recorded by a Trattner hydrophorograph (5) and an electrical drop recorder, respectively.

C. Cardiovascular Effects.—Carotid blood pressure was recorded by either an aneroid or mercury manometer in three dogs anesthetized with sodium pentobarbital, 25–30 mg./Kg., intraperitoneally. The responses to acetylcholine iodide, 0.01 mg./Kg., and of epinephrine, HCl 0.01 mg./Kg., intravenously were determined before and after administration of meperidine, 5 to 20 mg./Kg., intravenously.

Human Studies

Gastric Emptying.—Emptying of the human stomach was estimated by a method which essentially consisted of measuring the area of stomach which was filled with barium sulfate before and forty minutes after the intramuscular administration of 100 mg. of meperidine or 2 cc. of saline. Ten subjects received meperidine and 10 others normal saline.

RESULTS

A. Intestinal Activity.—Meperidine in doses of 0.5 and 1.0 mg./Kg. intravenously had no influence on the motility of the ileum or jejunum in 9 anesthetized dogs. When doses of 2.0, 4.0, 5.0, and 10 mg./Kg. were administered to these same animals, there was an appreciable increase in amplitude of contraction and usually some increase in rate of contraction and tone although these effects were not as marked as the increase in amplitude of contraction. The results obtained on total activity are summarized in Table I and a representative tracing is presented in Fig. 1. These results are in essential agreement with those of Gruber, Hart, and Gruber (2), Yonkman, Noth and Hecht (3), and Scott and his co-workers (1), and are quite similar to those ob-

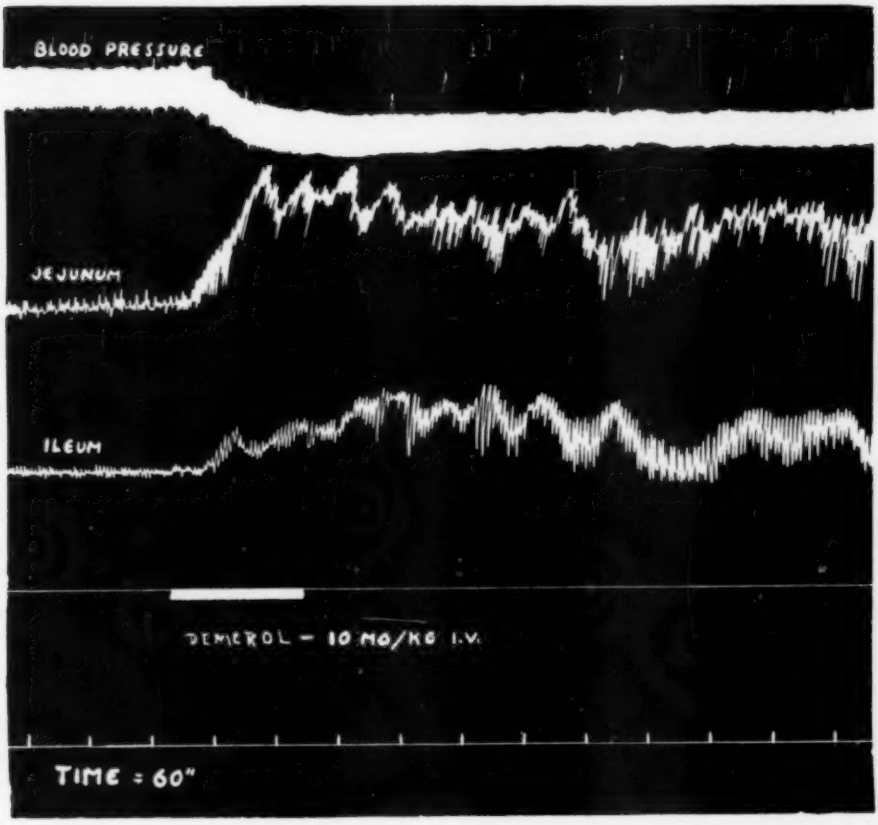


Fig. 1.—Response of small bowel to Demerol.

TABLE I.—AVERAGE RELATIVE EFFECTS OF MEPERIDINE ON INTESTINAL ACTIVITY OF THE ANESTHETIZED DOG

Dose, I. V., Mg./Kg.	Number of Observations	Amplitude of Contraction
0.5	1	0[a]
1.0	3	0
2.0	4	2+[b]
4.0	3	3+
5.0	4	3+
10.0	6	3+

[a] 0 = no change from control activity.
[b] + = increase from control activity.
Key: 2+ = moderate increase, 3+ = marked increase.

tained with morphine on the small intestine of most animals.

B. Extra-Hepatic Biliary Tract Activity.—In two experiments performed on the unanesthetized dog, meperidine, 3 mg./Kg., intravenously, increased the amplitude and number of contractions and the outflow of bile from the gall bladder. A typical record showing the effects of meperidine on the extra-hepatic biliary tract is shown in Fig. 2. These results are in keeping with the findings of Gaensler, McGowan, and Henderson (6) in man which indicated

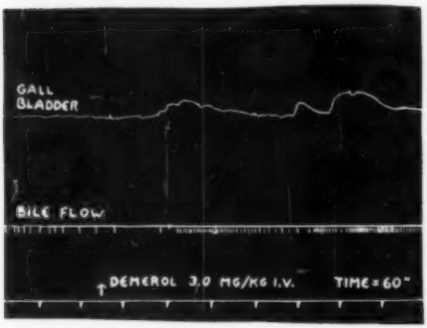


Fig. 2.—Effect of Demerol on the gall bladder.

that biliary pressure is increased by meperidine. They believe that the increase in pressure is due to duodenal spasm. In addition to this factor, it is necessary to recognize the stimulant effect on the extra-hepatic biliary tract itself.

C. Cardiovascular Effects.—Meperidine did not influence the acetylcholine-induced fall in arterial blood pressure in 3 anesthetized dogs (see Figs. 3 *A* and *B*). The response of these same animals to acetylcholine before and after the administration of a potent anticholinergic substance (β-piperidinoethyl phenyl-α-thienyl glycolate) is shown in Figs. 3 *B* and 3 *C*. Eisleb and Schaumann (7), in their pharmacologic evaluation of meperidine, stated that it possesses $^1/_{10,000}$ the activity of atropine in combating an acetylcholine-induced fall of arterial blood pressure. Meperidine itself produces vasodilation when administered intravenously and it is reasonable to expect that this could easily account for any decrease in vasodepressor response to acetylcholine which is only $^1/_{10,000}$ that of atropine.

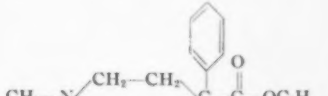
Gastric Emptying.—Meperidine reduced the emptying time of the stomach in 10 subjects so that 55 ± 11% of the barium sulfate originally in the stomach was still present 40 minutes later. The 10 control individuals who were subjected to exactly the same manipulations as those receiving meperidine retained only 24 ± 6% of the barium sulfate originally in their stomachs. A statistical evaluation of the data by Student's "t" test indicated that the probability of such an observation occurring due to chance was 1:50, thus indicating that these results are highly significant.

It may appear difficult to interpret the results of this experiment as indicating spasmogenic action on the intact human stomach; however, there are a number of lines of reasoning and experimental facts which will support such a conclusion. From similar observations on the intact human stomach, it appears that morphine produces a sequence of events which would bring about the same results as those found with meperidine. Morphine initially increases gastric motility which is followed by a cessation of activity as shown by Abreu, Dailey, Ould, Pickering and Reynolds (8). Since an agent such as tetraethylammonium bromide, a potent gastric antispasmodic, is known to increase the size of the stomach as indicated by roentgenographic studies (9), it would be expected that if meperidine were exerting any appreciable antispasmodic action, it would produce a similar picture. Measurements of the total area of the stomach do not support this conclusion (8).

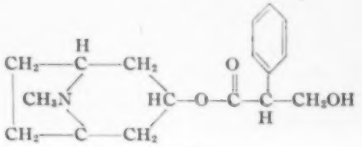
Batterman (10) has interpreted his balloon records of gastric activity as indicating an over-all depression of the stomach. By close inspection of his published records, one is led to the conclusion that meperidine produces an initial increase in gastric motility followed by a cessation of activity. In addition, his records during the period of arrested motility either show no decrease or an increase in tone. It is difficult under these circumstance to ascribe to meperidine any gastric antispasmodic action.

DISCUSSION

The structures of atropine and meperidine can be represented so that they appear similar.



Meperidine



Atropine

However, it has been shown by Blicke (11) and Lands, Nash, and Hooper (12) that atropine-like activity depends upon an amino alcohol group which meperidine does not contain. Although on casual inspection the structures may appear similar, the absence of an amino group in the alcoholic portion of the ester makes it unlikely that an atropine-like action can result from this structure. Duguid and Heathcote (13) reported a meperidine antagonism to acetylcholine depression of the isolated frog heart. However, the recent work of Scott and his coworkers (1) demonstrated that this observation did not hold for the intact mammalian heart. They have shown in trained, unanesthetized dogs that meperidine in doses of 20 mg./Kg. intraperitoneally resulted in a mean drop of cardiac rate from 72 to 48 per minute. In 2 of the animals given this dose of meperidine, tachycardia immediately resulted from the administration of atropine sulfate, 0.65 mg./Kg., intravenously. These results and the effects produced on the gastro-enteric tract are identical with those obtained with Methadone (1).

SUMMARY

1. Meperidine in doses ranging from 2.0 to 10 mg /Kg., intravenously increased the activity of the small intestine of the anesthetized dog.

2. In doses of 3 mg./Kg., intravenously, meperidine increased the motility of the gall bladder and bile flow of the unanesthetized dog.

3. There was no decrease in the blood pressure response to acetylcholine produced by meperidine in doses ranging from 5 to 20 mg./Kg., intravenously.

4. The emptying time of the intact human stomach is decreased by meperidine, 100 mg.,

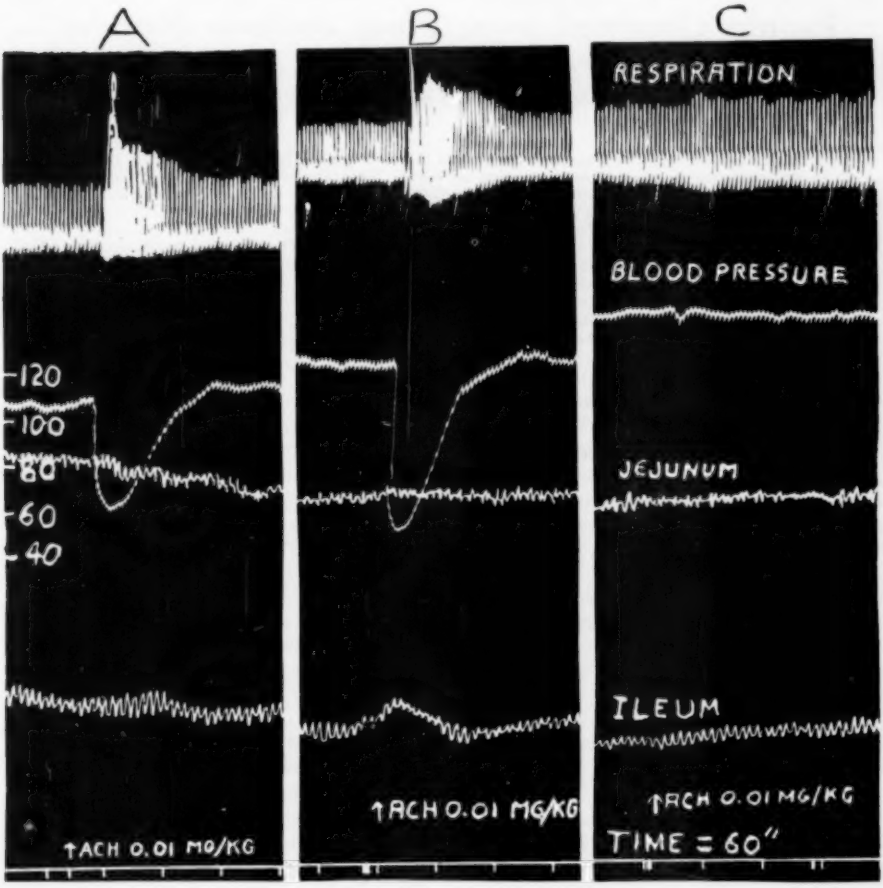


Fig. 3.—Cardiovascular effects of Demerol.

A, control response; *B*, response after 5 mg./kg. meperidine; *C*, response after ppt.

intramuscularly. A discussion of this aspect of action leads to the conclusion that this may not indicate antispasmodic activity.

5. Evidence is presented which makes it difficult to assign any atropine-like activity to meperidine.

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A Preliminary Phytochemical Investigation of the Tannin Obtained from *Pinus caribaea* Morelet*

By FRANK W. BOPE† and OLE GISVOLD‡

A new and simple technique was developed in order to remove the water soluble substances from the tannin found in *Pinus caribaea*. This tannin exhibited all the chemical and physical properties usually attributed to phlobatannins. The so-called "phlobaphene test" when applied to certain derivatives gave a red color which indicated the development of a chromophoric group whose visible absorption spectrum was in the range of that exhibited by the anthocyanins. The colored compounds could be smoothly reduced to a colorless or nearly colorless compound which in appearance was much like the original compound. Analyses of several derivatives indicated that $C_{15}H_{14}O_6$ residues might be present in the tannin molecule. The ultraviolet absorption spectrum of the original tannin and its derivatives was at about 2775 Å. which is in the region exhibited by several other naturally occurring phlobatannins and some anthocyanins.

THE LEATHER INDUSTRY is constantly consuming large quantities of both natural and synthetic tannins, and as a result the readily available sources of vegetable tannins are rapidly being depleted. This, of necessity, demands that all possible sources of future supply be investigated and utilized as far as possible.

The discovery of the presence of appreciable quantities of a natural vegetable tannin in the slash pine tree, *Pinus caribaea* Morelet, indicated the need for a study of the amount present, the physical properties, and the chemical properties of the tannin.

It is common knowledge today that one of the greatest problems involved in tannin chemistry is that of obtaining pure or homogeneous materials. Extraction of crude tannin from the bark or wood of various plants almost always results in extraction of some other plant products such as sugars, plant pigments, etc. Most tannins are amorphous substances and are exceedingly difficult to purify. The constituents of most of them are still unknown. Most tannins belong to the phlobatannin class of condensed tannins. They are related to the normal plant pigments of the benzopyran type and their nearest analogues are the flavans. The most frequent occurring nucleus of the flavan type is catechin.

According to Russell (1) phlobatannins must be built on the catechin model. Russell and coworkers (2, 3) prepared a number of synthetic compounds which they believed to be flavpinacols and they reported them to be qualitatively indistinguishable from typical natural phlobatannins. Russell, Todd and Wilson (4) measured absorption spectra of some synthetic flavpinacols and of some typical natural phlobatannins. The

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results supported the view that phlobatannins were polyhydroxy-flavpinacols.

In 1935 Freudenberg and Steinbrunn (5) stated that what Russell considered as a synthetic imitation or form of hemlock tannin (his dimer flavpinacol) doubtless possessed higher molecular weight. They regarded it as premature to assign any constitutional structure to hemlock tannin.

The object of this work was to investigate the tannin obtained from *Pinus caribaea* Morelet with the view of determining the amount of tannin present and studying its physical and chemical properties.

A preliminary pharmacological investigation of *Pinus caribaea* phlobatannin involving acute toxicity studies and the local treatment of experimental burns with a comparison to tannic acid has been carried out and will be published elsewhere.

EXPERIMENTAL

Collection and Extraction of Bark.—The inner bark or phloem of *Pinus caribaea* was collected through the courtesy of the Forest Division of the United States Department of Agriculture and was shipped from the Olustee Experimental Forest, Olustee, Fla., in the spring of 1947. The bark was air dried and ground to a No. 20 powder in a Jacobson laboratory mill.

The bark (3200 Gm.) was defatted by extraction with ether for eighteen hours in a continuous extraction-type metal percolator. The defatted bark weighed 3075 Gm. It was macerated for twenty-four hours in large evaporating dishes with 13,500 cc. of 95% alcohol. The macerated bark was packed in a large stone percolator and was allowed to stand for twenty-four hours. Percolation was then allowed to proceed slowly, more menstruum being added as needed. The total amount of 95% alcohol used to macerate and extract the defatted bark was 22,500 cc. The alcoholic extract was concentrated to dryness by vacuum distillation at a very low temperature. The yield of crude tannin was 753 Gm. or about 23% of the original dry, milled bark.

Purification.—The main task of purification was the removal of sugars. An attempt was made to purify the tannin through precipitation by means of lead acetate, but the process proved to be difficult to manipulate because of the colloidal nature of the lead-tannin precipitate and the length of time involved in washing out all the sugar.

Purification by precipitation with sodium chloride as described by Russell (1) was an improvement over the lead acetate method as far as ease of manipulation was concerned, but it proved to be very inefficient, making it necessary to find another method of removing sugars.

It was found that tetrahydrofuran would not extract sugars from aqueous solutions, but that this phlobatannin was very soluble in tetrahydrofuran and could be extracted from aqueous solution by means of a salting-out process using sodium sulfate. The crude tannin (100 Gm.) was dissolved in 800 cc. of hot water and was allowed to stand at room temperature for at least twenty-four hours, a small amount of chloroform being added as a preservative. Some red amorphous insoluble materials (4.3 Gm.) deposited and were removed by filtration. The filtrate was partially reduced or decolorized by shaking with sodium hydrosulfite, and was then extracted with 400 cc. of tetrahydrofuran in a separatory funnel. To this mixture was added an excess of sodium sulfate which effected a salting out, the tannin and tetrahydrofuran separating as the upper layer and the water, salt, and sugars as the lower layer. The aqueous layer was removed, more water was added and the salting out process was repeated. This process was continued until the aqueous layers failed to give positive tests for sugars with Molisch Reagent. The tetrahydrofuran was then removed by vacuum distillation and the resulting purified tannin weighed 42.6 Gm. or 42.6% of the original crude tannin.

Identification of Sugar.—The sugar present in the crude tannin was identified as glucose through formation of its osazone. Microscopic comparison of the crystals with those of glucosazone, rate of formation, and melting point indicated the sugar osazone was identical with glucosazone.

The sugar present gave a positive test with Fehlings solution and a negative Seliwanoff test (6) which indicated the absence of ketoses.

Properties of the Tannin.—The original crude tannin was a light red amorphous powder soluble in water, methyl alcohol, and ethyl alcohol; and insoluble in ether, benzene, and petroleum ether. It darkened in alkali and upon exposure to air it absorbed moisture. It was polymerized by heat.

The purified tannin was a slightly darker red amorphous powder with properties similar to those of the crude tannin except that it was slightly less soluble in water. It had no melting point but gradually decomposed above 195°. Its behavior when boiled with dilute mineral acids was characteristic of typical phlobatannins; it produced a bright red solution and a red precipitate of insoluble phlobaphenes.

The purified tannin was subjected to a number of qualitative tests as outlined by Russell (1). With ferric chloride it produced a dark green color; with gelatin a white precipitate was produced; lead acetate produced a light precipitate which was soluble in acetic acid; with boiling dilute HCl it produced a red solution and a red precipitate; with bromine water it gave an immediate heavy yellow precipitate; and with potassium dichromate it gave a brown precipitate.

Attempts to reduce the purified tannin with platinum black or with palladium on charcoal under 40 lb. pressure of hydrogen were unsuccessful. No apparent reduction was obtained. By refluxing 1 Gm. of tannin with 5 Gm. of zinc dust and 1 cc. of glacial acetic acid in alcoholic solution for ten minutes some decolorization or reduction occurred. This decolorization was not permanent, however, and attempts to reduce or decolorize larger amounts were unsuccessful.

Methylation with Diazomethane.—The first attempted methylation with diazomethane involved a partially reduced tannin. Ten grams of purified tannin were dissolved in 150 cc. of methyl alcohol

and the solution was partially reduced by the addition of sodium hydrosulfite. It was then methylated by diazomethane prepared from 30 Gm. of N-nitrosomethylurea in the usual manner. When an excess of diazomethane remained overnight, a light-colored precipitate was obtained that was removed by filtration, washed, and dried. The yield was about 9 Gm. of an amorphous methyl ether that had no melting point but darkened or decomposed slowly above 220°.

Better methylated products were obtained when no attempts were made to first reduce the tannin. Five grams of purified tannin was dissolved in 200 cc. of anhydrous methyl alcohol and the solution was methylated with an ethereal solution of diazomethane prepared in the usual manner. It required the diazomethane from 65 Gm. of N-nitrosomethylurea to maintain an excess of the methylating agent for twenty-four hours. The resulting pale yellow amorphous precipitate was filtered out, washed with cold water and dried in a vacuum desiccator over NaOH and then for six hours over P_2O_5 in the Abderhalden drying pistol. The yield was 3.2 Gm. of methylated tannin which decomposed at 228–230° without melting. The yield was increased to 4.7 Gm. by dilution of the filtrate with 300 cc. of cold water. Attempts to purify or crystallize the methylated tannin were of no practical value.

Anal.—Calcd. for $C_{20}H_{20}O_8(OCH_3)_6$: C, 65.24; H, 5.78. Found: C, 62.38, 62.70; H, 5.68, 5.88.

Methoxyl.—Calcd.: 28.10. Found: 28.12, 28.19.

The methylated tannin produced a cherry-red solution when boiled for a few minutes with 4 drops of concentrated HCl in 10 cc. of alcohol (a positive phlobaphene test), and it produced no color with ferric chloride test solution.

Methylation with Dimethyl Sulfate and Alkali.—Because of the instability of the phlobatannin to alkali, it was necessary to methylate the tannin with dimethylsulfate and alkali in an atmosphere of nitrogen. Two grams of purified tannin was dissolved in 100 cc. of water and the resulting red-colored solution was reduced by addition of zinc dust and a few drops of concentrated HCl. The zinc dust was removed by filtration and the solution was placed in a three-necked flask equipped with a mechanical stirrer, a separatory funnel, and a tube leading from a nitrogen tank. In an atmosphere of nitrogen the solution was made alkaline by the addition of 5 cc. of 50% KOH. Some precipitate resulted and then 5 cc. of dimethyl sulfate were added at once. The mixture was allowed to become acid and then was made alkaline slowly by the dropwise addition of 5 cc. of 50% KOH. It was made alternately acid and alkaline in the same manner until a total of 35 cc. of dimethyl sulfate had been added. The total amount of 50% KOH used was 45 cc., leaving the reaction mixture strongly alkaline at the end. The total time for the addition of the reagents was about four hours and the temperature was not allowed to exceed 60°.

The methylated product was removed by filtration and when washed and dried it weighed 1.7 Gm. It did not melt but gradually darkened above 200°.

In order to obtain complete methylation, the product was remethylated in an alcoholic medium instead of an aqueous medium. A total of 20 cc. of dimethyl sulfate and 25 cc. of 50% KOH was added in small portions as previously described. The final methylated product after one precipitation from alcohol still gave no melting point but decomposed above 200° as before. It gave a negative ferric chloride test, but a positive phlobaphene test when boiled for twelve minutes. The methoxyl content was raised to 33.56% and 33.59% methoxyl by this method of methylation.

Methylation with Methyl Iodide and Silver Oxide.—Methyl iodide catalyzed by moist silver oxide was employed on the tannin previously methylated by diazomethane in an attempt to methylate a possible sterically hindered group. The procedure was similar to that described by Purdie (7). The product obtained was a light red amorphous compound which still had no melting point but decomposed above 160°. Methoxyl determinations showed 29.24% and 29.23% methoxyl indicating very little, if any, additional methylation.

Acetylation.—Acetylation by means of pyridine and acetic anhydride produced slightly pink amorphous products which did not melt but decomposed at about the same temperature as the original tannin.

Reductive Acetylation.—Acetylation with acetic anhydride and sodium acetate in the presence of zinc dust produced almost colorless acetates. The purified tannin (4 Gm.) was dissolved in 90 cc. of acetic anhydride by heating under a reflux condenser. To the resulting red solution were added 5 Gm. of zinc dust and 1 cc. of glacial acetic acid and the mixture was refluxed for ten minutes, producing a light green-yellow clear solution. The addition of glacial acetic acid and zinc dust was repeated and the mixture was refluxed another ten minutes. A third addition of these reagents was followed by 0.5 Gm. of fused sodium acetate and the mixture was refluxed for forty minutes. At the end of this time the mixture was filtered hot and the filtrate was poured slowly with stirring into 400 cc. of ice water. After standing overnight the almost colorless amorphous acetylated tannin was removed by filtration, washed thoroughly with water, and dried in the usual manner. The yield was 5 Gm. and the compound had a wide melting-point range of 103–130°. Several purifications from alcohol did not alter the melting point although an insoluble fraction amounted to 0.7 Gm. and decomposed above 170° without melting.

In order to minimize polymerization, condensation, or dehydration an acetylation was carried out at 50–55°. The procedure was similar to that just described except that the reaction was kept at 50–55° for 11 hours and then at room temperature overnight. Two grams of purified tannin yielded 2.1 Gm. of acetylated tannin which melted at 108–130°.

The acetylated tannin gave a negative test with ferric chloride and no phlobaphene test when boiled for fifteen minutes with dilute mineral acids. The addition of more acid and prolonged boiling produced a pink color indicating phlobaphene formation.

Anal.—Calcd. for $C_{20}H_{18}O_4(OAc)_8$: C, 60.39; H, 4.59. Found: C, 59.45, 59.25; H, 5.12, 5.01.

Acetyl Value.—Calcd. for $C_{20}H_{18}O_4(OAc)_8$: 37.64. Found: 37.49, 37.88.

Methoxyl determinations were carried out on the acetylated tannin to determine if any methoxyl groups were originally present in the tannin. The results showed 1.53% and 1.80% methoxyl.

Saponification of the Acetate.—About 0.3 Gm. of acetylated tannin was dissolved in 15 cc. of alcohol

with the aid of heat. In an atmosphere of nitrogen, 20 cc. of 2.5% alcoholic KOH were added drop by drop with frequent shaking. After twenty-four hours in an atmosphere of nitrogen the solution was made slightly acid with dilute HCl and 15 cc. of water were added. The alcohol was then removed by vacuum distillation leaving an amber-red solution. The tannin recovered in this manner by saponification gave a dark green color and green precipitate with ferric chloride, a white precipitate with gelatin and a positive phlobaphene test.

Fractionation of the Acetylated Tannin.—One gram of acetylated tannin was extracted with 50 cc. of ether for one hour under a reflux condenser. A second extraction was then carried out in the same manner. Evaporation of the ether solutions yielded a few milligrams of residue which, when dried, melted at 50–60°. Its properties were similar to the original acetate, and when saponified in an atmosphere of nitrogen as described, it gave positive tests with ferric chloride, gelatin solution, and boiling dilute HCl (phlobaphene reaction).

Reductive Acetylation of the Methylated Tannin.—A reductive acetylation of the tannin previously methylated with diazomethane was carried out in the usual manner. The acetylated methyl ether did not melt but gradually decomposed above 235°. It gave a negative test with ferric chloride, not producing a positive phlobaphene color until boiled over twenty minutes with dilute HCl.

Acetyl Value.—Calcd. for $C_{20}H_{18}O_4(OCH_3)_6$-$(OAC)_2$: 11.51%. Found: 16.36%.

Lead Tetraacetate Oxidation.—If Russell's flavpinacol structure for phlobatannins was correct, it should be possible to cleave the phlobatannin molecule with lead tetraacetate. A total of 14 separate reactions were carried out using a 0.05 molar solution of lead tetraacetate in glacial acetic acid.

A carefully weighed amount (about 0.45 Gm.) of acetylated (or, in two cases, methylated) tannin was dissolved in 25 cc. of glacial acetic acid in a 250-cc. flask. To this solution was added 30 cc. of 0.05 molar lead tetraacetate and in some cases 5 cc. of methyl alcohol, in others 5 cc. of water. The mixture was kept at 50–55° for nine hours and then at room temperature for fifteen hours. The resulting reaction mixture was then titrated according to the procedure of Criegee (8) using sodium thiosulfate. The amount of lead tetraacetate consumed was 1.14 moles per mole of acetylated tannin used.

Many attempts were made to isolate cleavage products from all the reactions but none could be isolated other than recovery of what apparently was the original acetate.

Phlobaphene Study.—Purified tannin when dissolved in 10 cc. of alcohol and boiled with 4 drops of concentrated HCl, produced a bright red solution. This solution was decolorized by zinc dust, and when the red solution was shaken with sodium bicarbonate it darkened to a purple-red color. Sulfuric acid produced the phlobaphene color as well as HCl.

When the above test was carried out in water, a red insoluble material deposited. This red phlobaphene was slowly soluble in alcohol to produce a cherry-red solution which was completely decolorized by zinc dust. The decolorized solution was diluted with water and the alcohol was removed by vacuum distillation to produce a small amount of red insoluble material again.

An alcoholic solution of the tannin methylated by diazomethane, when boiled with 4 drops of HCl, produced a positive phlobaphene color in four minutes. The red color was completely decolorized by zinc dust and after removal of the zinc, heating with one drop of HCl restored the red color. The original red solution when shaken with sodium bicarbonate changed to a light purple color, and with excess bicarbonate it completely decolorized. Sodium hydrosulfite also decolorized the red solution.

The tannin methylated with dimethyl sulfate and alkali produced the red phlobaphene color much slower (twelve minutes).

The acetylated tannin when dissolved in alcohol and boiled with 4 drops of HCl produced no red color in fifteen minutes. The addition of more acid and prolonged heating produced a pink color which was decolorized by zinc dust and was restored after removal of the zinc by heating. The decolorization by zinc dust was not permanent, but the color slowly returned. Other reactions of the phlobaphene of the acetate were similar to those described for the methyl ether above.

Absorption Spectra.—Solutions containing 50 mg. of the desired tannin compound in 50 cc. of 95% alcohol were diluted ten times for use in measuring absorption spectra. The tannin compounds used were: purified tannin, reduced purified tannin, acetylated tannin, tannin methylated by diazomethane, tannin methylated by dimethyl sulfate, and the phlobaphened solutions of both methylated tannins. The solutions were measured in the Beckman Model DU Photoelectric quartz spectrophotometer using the ultraviolet sensitive phototube. In the ultraviolet region all the compounds showed maximum absorption at 2775 Å. Readings in the visible region of the spectrum were made on the Coleman Electric Spectrophotometer. There was no absorption in the visible region for any of the compounds except the phlobaphened methyl ethers. Both of the latter showed maximum absorption at 5350 Å.

SUMMARY AND CONCLUSIONS

A natural phlobatannin has been extracted from the inner bark of the slash pine tree, *Pinus caribaea* Morelet.

A new method for purification of the crude tannin involving the use of tetrahydrofuran has been presented. The sugar component of the crude tannin extract was identified as glucose.

The properties of the phlobatannin have been determined. It is an amorphous tannin. A number of reactions have indicated that it is qualitatively indistinguishable from other typical natural phlobatannins.

The phlobatannin has been methylated with diazomethane to produce an almost colorless amorphous methyl ether. Methoxyl determinations have indicated three hydroxyl groups per $C_{15}H_{14}O_6$ residue capable of methylation with diazomethane. The methoxyl content was increased by the use of dimethyl sulfate and alkali, indicating the probability of four hydroxyl groups per $C_{15}H_{14}O_6$ type residue capable of methylation.

Reductive acetylation of the phlobatannin produced an almost colorless amorphous acetate. Analytical results showed four hydroxyl groups capable of acetylation per $C_{18}H_{14}O_8$ residue or eight on the basis of Russell's suggested flavpinacol type structure.

The acetylated tannin has been saponified to yield the original tannin. Fractionation by extraction with ether produced two different melting acetates.

A reductive acetylation of the tannin previously methylated by diazomethane succeeded in acetylating an additional hydroxyl group.

Attempted cleavage of certain derivatives of the phlobatannin from *Pinus caribaea* Morelet with lead tetraacetate indicated that a simple flavpinacol structure was probably absent, although quantitative amounts of lead tetraacetate were consumed.

A study has been made of the formation of phlobaphenes. It has been demonstrated that certain substituted hydroxyl groups influence the rate of formation of the phlobaphene, due possibly to steric hindrance, but also that free hydroxyl groups are not essential for the reaction. It is, therefore, fair to conclude that the splitting out of water is not the primary step in the production of the phlobaphene. It may result secondarily.

Absorption spectra measurements were very similar to those of certain well-known anthocyanins. The anthocyanin structure may be basic or fundamental in the production of the red color in the phlobaphene reaction.

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A Phytochemical Investigation of the Kelps *Cymathaere Triplicata, Hedophyllum Sessile,* and *Egregia Menziesii**

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The phytochemical investigation of three northwest marine algae is presented including a survey of the plants for their vitamin content and antibiotic properties. The results obtained are presented with a summary indicating possible usefulness of the plants.

SEA WEEDS and their products appeared as food as early as the 17th century in the history of the Japanese. For many years, agar, a gelatinous substance obtained from *Gelidium, Gracilaria,* and other genera of *Rhodophyta,* has been a common food for both the Japanese and Chinese, and it was from them that its use spread to the East Indies and later to Europe. The French used it for food in the 18th century and called it "mousse de chine." The use of agar in culture media, in medicine as a laxative, and its application in the manufacture of certain pharmaceutical preparations are well known. The Japanese also cultivate porphyra, another of the red algae, on their tidelands (1), the annual harvest being worth several million dollars. The plants are dried and flaked or powdered for the market forming a food product called Kombu. The kelps of the United States, mostly members of the brown algae or *Phaeophyta,* have found application in medicine as valuable sources of minerals and vitamins.

The red and brown algae are the important sources of commercially valuable or potentially valuable substances such as agar, carrageenin, funorin, and algin. These are all mucilaginous substances of a colloidal nature and are generally spoken of as phycocolloids. Algin is used as a food thickener, a plastic, a sizing material, an

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emulsifier, a water softener, and an excipient in pharmacy.

Of the kelps under consideration, *Cymathaere triplicata*, *Hedophyllum sessile*, and *Egregia Menziesii*, all are members of the brown algae and none has been previously studied chemically with the exception of *Egregia Menziesii*, the chemistry of the entire plant of this species having been investigated by Hoagland (2).

This investigation was undertaken to ascertain the proximate constituents, the vitamin concentration, and the mineral content of the ash, for the purpose of determining the possible value of these plants as fertilizers or food supplements. A study was made of the content of algin, laminarin, and mannitol because of their potential importance, and the fact that little is known concerning the quantities of these substances in the plants under consideration. A preliminary survey was made on the plants to determine possibilities of the presence of any antibiotic substances since they are of great importance medicinally.

EXPERIMENTAL

Collection and Preparation of the Material.—The samples used in this study were collected in late August, 1946, at False Bay near Friday Harbor, Wash. After identification by Dr. George B. Rigg of the Botany Department, University of Washington, the plants were transferred to the drying chambers in the greenhouse of the College of Pharmacy Drug Garden and were hung on wires in such a manner that there was free passage of air between the plants. The air, at a uniform 27° temperature, was kept circulating by fans in the drying chambers. A few of the plants were dried in the greenhouse proper under the same conditions except that the temperature was maintained at 27° only during the daytime. The plants were turned occasionally to prevent decomposition from contact and to promote proper drying. After the plants were dried they were ground to a No. 60 powder and stored in glass jars sealed with rubber-seated screw tops.

Two other collections of the plants were made to obtain fresh material at two different seasons for the vitamin assays. The collections, one in June and the other in September, 1947, were made in the same locality. The two collections of fresh plants were stored at 0° in a freezing unit at Friday Harbor, Wash., within two hours after gathering. After freezing for twenty-four hours they were packed in ice, properly insulated, and moved to Seattle where they were placed in a freezing unit at 0°. The vitamin assays were made on this material within six months after collection. Preceding the vitamin analyses on the fresh frozen plants, they were warmed to room temperature, dried as thoroughly as possible, and well minced before weighing. No attempt was made to separate any of the plant parts, therefore the analyses represent the amounts in the entire plant. All determinations were made in duplicate except the vitamin assays which were made in triplicate, and were repeated when necessary until checks were obtained.

Partial Proximate Analysis.—In the determinations that follow methods as outlined in the A. O. A. C. were used in every case and the results, calculated on a moisture-free basis, are presented in Tables I and II.

TABLE I.—PARTIAL PROXIMATE ANALYSIS OF THE KELPS

	Hedophyllum sessile, %	*Egregia Menziesii*, %	*Cymathaere triplicata*, %
Moisture (dried plants)	8.84	8.37	7.12
Moisture (fresh plants)	54.86	74.80	69.81
Crude fiber	3.54	10.30	6.10
Organic and ammoniacal nitrogen	2.71	2.57	1.68
Starch	Negative	Negative	Negative
Reducing sugars	Negative	Negative	Negative
Sucrose	Trace	Trace	Trace
Ash	35.27	36.25	27.97

TABLE II.—COMPOSITION OF THE ASH OF THE KELPS

	Hedophyllum sessile, %	*Egregia Menziesii*, %	*Cymathaere triplicata*, %
Water-soluble ash	27.71	23.46	23.78
Water-insoluble ash	7.56	12.79	4.19
Acid-insoluble ash	2.01	6.88	0.11
Sulfates	11.24	6.14	11.06
Calcium oxide	4.46	3.35	3.72
Magnesium oxide	4.34	1.99	3.33
Phosphates as P_2O_5	2.52	5.05	5.60
Potassium	10.43	9.40	9.86
Sodium	13.57	11.39	10.51
Chlorine	32.59	27.62	31.44
Bromine	Trace	Trace	Trace
Iodine	0.09	0.06	0.09
Alkalinity of water-soluble ash (cc. 1 *N* HCl/100 Gm.)	4.53 cc.	2.99 cc.	7.56 cc.

Extractions.—Samples of the dried plant were extracted selectively in a Soxhlet apparatus by the continuous extraction method using petroleum ether, ether, chloroform, and ethyl acetate as solvents in the order named. The extracts were evaporated, dried to constant weight at 100°, and the results are presented in Table III.

TABLE III.—RESULTS OF SELECTIVE EXTRACTION OF THE KELPS

Kelp	Petroleum Ether Extract, %	Ether Extract, %	Chloroform Extract, %	Ethyl Acetate Extract, %
Hedophyllum sessile	1.25	0.71	1.30	1.01
Egregia Menziesii	0.74	0.39	1.00	0.34
Cymathaere triplicata	3.39	0.51	0.64	0.44

Alginic Acid.—In a study on a number of species of kelp, Dillon and McGuiness (3) found a seasonal variation of algin content, the percentage being high in early spring and low in September.

The usual method for the preparation of algin is to treat the kelp with a solution of sodium carbonate; this forms the soluble sodium alginate which can be separated from the plants by filtration. The filtrate is then treated with a strong mineral acid and the liberated, insoluble alginic acid is collected on a filter.

In this study 50-Gm. samples of the dried ground plant were mixed with 4 L. of water and 50 Gm. of sodium carbonate. This mixture was heated to 80° for fifteen minutes, diluted to 12 L., and filtered. Forty cubic centimeters of 30% calcium chloride solution per liter of filtrate was placed in a container and the mixture was added with constant stirring. The resulting precipitate was collected, made up to about 12 L. with water, and the fibers broken up as much as possible. Hydrochloric acid was then added until the *p*H became stabilized at 1.4. After standing twenty minutes, the solid materials were screened off, hydrochloric acid and water were again added to bring the *p*H to 1.6, and the mixture was again allowed to stand for twenty minutes. This process was repeated, and the material was screened and washed with water until the wash water had a *p*H of 2.3 to 2.5. Concentrated ammonium hydroxide was added, mixed well, and the paste was spread out to dry.

	Ammonium Alginate, %
Hedophyllum sessile	15.79
Egregia Menziesii	13.09
Cymathaere triplicata	12.47

Laminarin.—A modified method combining some features of the methods of Barry (4), and of Colin and Ricard (5) was used for the separation of laminarin. In a study by Dillon and McGuiness (3) on the content of laminarin in kelp, it was found that the variations are substantially opposite to those of the algin content. The concentration of laminarin was found to be highest in the winter and lowest in spring and summer.

Approximately 300-Gm. samples of the fresh plant were used from the June collection. These were covered with 5% hydrochloric acid, allowed to stand forty-eight hours, filtered, and the process repeated a second time. The combined precipitates were taken up with hot water and the solution was treated with barium hydroxide in sufficient amount to give a 2% solution. This mixture was filtered and ethanol was added to the filtrate until the alcoholic content was 75%. Upon standing twenty-four hours, a flocculant precipitate of laminarin was obtained, which was separated and collected by centrifuging. The material obtained was identified by hydrolyzing with 5% hydrochloric acid and preparation of the glucosazone, which was identified microscopically and by its melting point, 205°.

	Laminarin, %
Hedophyllum sessile	0.37
Egregia Menziesii	1.66
Cymathaere triplicata	0.90

Mannitol.—Five-hundred-Gm. samples of the dried, ground plant were extracted for twelve hours with petroleum ether. The extraction was continued with 95% alcohol from which a large yield of crystals formed on standing overnight. These were filtered off, washed with boiling ether, then with alcohol, recrystallized from boiling alcohol, washed again with alcohol, and the crystals were dried in a desiccator. The melting point of the crystals was 166–168° which is identical with that of mannitol.

	Mannitol, %
Hedophyllum sessile	0.50
Egregia Menziesii	0.50
Cymathaere triplicata	0.50

Vitamin Determinations.—As previously stated all vitamin determinations were made on samples of the dried and fresh frozen plants collected at two different seasons. The results of all determinations are shown in Table IV.

TABLE IV.—VITAMIN CONTENT OF THE KELPS

Vitamin	*Hedophyllum sessile*	*Egregia Menziesii*	*Cymathaere triplicata*
Thiamine, mg./Gm.			
Spring collection, fresh	1.23	0.60	0.24
Fall collection, fresh	2.18	0.40	0.63
Dried plant	2.65	0.25	0.50
Riboflavin, γ/Gm.			
Spring collection, fresh	8.71	15.92	5.36
Fall collection, fresh	6.68	6.88	7.16
Dried plant	8.55	4.36	2.77
Niacin, γ/Gm.			
Spring collection, fresh	47.19	52.14	39.41
Fall collection, fresh	35.00	45.37	28.81
Dried plant	69.42	83.56	60.29
Pantothenic Acid, γ/Gm.			
Spring collection, fresh	5.54	40.60	10.59
Fall collection, fresh	3.02	4.54	5.91
Dried plant	54.59	50.74	48.46
Carotene, γ/Gm.			
Spring collection, fresh	8.86	15.92	9.94
Fall collection, fresh	19.05	23.89	15.24
Dried plant	6.85	2.18	3.41
Ascorbic Acid, γ/Gm.			
Spring collection, fresh	26.0	49.0	54.0
Fall collection, fresh	33.0	42.0	60.0
Dried plant	26.0	60.0	133.0

Thiamine.—The thiamine determinations were made by the thiochrome method, A. O. A. C. (6), and the results were calculated to mg. per Gm. on a moisture-free basis.

Riboflavin.—The A. O. A. C. (6) microbiological method was used. The riboflavin was extracted from the samples in the prescribed manner and a series of standard solutions was set up along with triplicate series of the unknown. Basal medium stock solution was added to each tube of the series,

brought to the same volume, sterilized, inoculated with a suspension of *Lactobacillus casei*, and incubated at 37° for seventy-two hours. The contents of each tube were then titrated with 0.1 *N* sodium hydroxide solution, using bromthymol blue as the indicator.

A standard curve was prepared from the riboflavin standard titration by plotting the average of the titration values expressed in cc. of 0.1 *N* sodium hydroxide solution, for each level of the riboflavin standard used, against micrograms of riboflavin contained in the respective tubes. The riboflavin content of the unknown solution in each tube was obtained from this curve, and the riboflavin content in each cc. of the test material for each set of tubes was calculated. The riboflavin content of the test material was calculated from the average of the values obtained from not less than three sets of these tubes which did not vary by more than 10% from the average.

Niacin.—The A. O. A. C. (6) microbiological method was used, a procedure which is similar to that for riboflavin, except that the inoculum consists of *Lactobacillus arabinosus*. The nicotinic acid content in the test material was calculated from the average titration values obtained from not less than 6 of the tubes which did not vary by more than 10% from the average.

Pantothenic Acid.—The microbiological method (7), using *Lactobacillus arabinosus*, was employed. The procedure is essentially the same as that used in the two previous assays. The pantothenic acid content of the test material was calculated from the average of the values obtained from not less than 4 tubes whose deviation from each other was not more than 10%.

Carotene.—The A. O. A. C. (6) method was used in which the sample was refluxed with alcoholic potassium hydroxide and the carotene extracted with petroleum ether. The impurities were adsorbed on magnesium carbonate in an adsorption tower and the carotene was determined in the photoelectric colorimeter against known concentrations of purified carotene. Colorimeter readings were plotted against concentrations of carotene and the curve was used for calculating the amount of carotene in the sample.

Ascorbic Acid.—The A. O. A. C. (6) method was employed in which aliquots of the prepared solutions of the samples were titrated with indophenol solution to a light rose-pink color.

Antibiotic Properties.—The purpose of this study was to survey the plants under investigation for the presence of antibiotic substances which could form the basis of further research in the isolation and characterization of any active substances present. The plants were subjected to standard methods which have previously been used and found effective. No attempt was made to isolate or identify any antibiotic substances.

Since the plants used in this survey were brown algae, a method was chosen similar to that used on algae by Pratt, Daniels, and co-workers (8), who extracted chlorellin from a nutrient solution in which *Chlorella vulgaris* and *C. pyrenoidosa* had been grown.

In the following tests, the organism used was *B. subtilis* as a spore suspension in broth culture (9). The culture was made by seeding the broth with the bacteria and incubating seven to eight days with constant agitation. The resulting culture was pasteurized at 60° for thirty minutes resulting in a spore suspension that could be used to seed plates of nutrient agar. An approximately constant concentration of spores was maintained by using the same volume of culture to seed the agar plates. The cooled liquid nutrient agar was seeded with the broth culture, 25 cc. of broth to 200 cc. of agar, poured into Petri dishes, refrigerated one hour to set the plates, and then the various extracts from the plants were applied by soaking $^1/_2$-in. filter paper disks with the solution and applying three to each plate. The plates were incubated for twelve to fourteen hours at 37°, and then examined for appearance of zones of inhibition around the disks. The whole operation is essentially that of the filter paper disk penicillin assay method given by Foster and Woodruff (9). In each case suitable controls were set up using the extracting material. Tests were also made in the same manner with sea water obtained from the University of Washington Department of Oceanography and checks were run using penicillin with a strength of 100 units per cc.

The first test was run on the plants by taking the dried, ground plant and applying about 0.1 Gm. to the spore suspension agar plates. After incubation, the plates were examined for inhibition.

The next test was to treat 10 Gm. of the dried, ground plant with 100 cc. of water and 200 cc. of ammonium sulfate solution, heating the mixture to a temperature of 80° and filtering. After cooling, the *p*H was checked but not adjusted since in every case the solution was acid (*p*H about 6). The filter paper disks were then impregnated with the filtrate, placed on the seeded agar plates, incubated for twelve to fourteen hours, and examined for zones of inhibition.

The procedure employed by Berger (10) for purification of penicillin was used as the third method. An aliquot from 300 cc. of the filtrate, obtained as given above, was extracted with butyl alcohol, the butyl alcohol was then shaken out with petroleum ether, and then the petroleum ether was extracted with 2% sodium bicarbonate solution. Amounts were used in each step to give a ratio of 100 cc. of solvent to 100 cc. of extractive. The natural *p*H of the plants was so close to that recommended by Berger (*p*H 6.4) that no adjustment was made. The sodium bicarbonate solution was used to saturate the filter paper disks which were applied and read as before. The sodium bicarbonate solution was also concentrated to double strength by evaporation, *in vacuo*, at 60° and tested for its antibiotic properties.

The method of Pratt, Daniels, *et al.*, (8) was modified as follows: 100 cc. of ammonium sulfate solution was extracted with 150 cc. of chloroform in divided portions. The chloroform extracts were combined, evaporated *in vacuo*, the residue taken up in water and tested on the spore suspension agar plates. Chloroform was the only solvent used. The results of the various tests are shown in Table V.

In order to ascertain whether the inhibitory activity of *Cymathaere triplicata* was similar to that of known antibiotics and not due to some factor in sea water the following control tests were made: sea water with negative results and penicillin 100 units per cc. which produced 16- to 18-mm. zones of inhibition.

DISCUSSION

In the three kelps investigated, *Cymathaere triplicata*, *Hedophyllum sessile*, and *Egregia Menziesii*, algin, laminarin, and mannitol were found in amounts approximately equal to that of similar kelps. However, the quantities obtained can only represent minimum limits, since in any extraction procedures involving large amounts of crude material and reagents, under laboratory conditions, inevitable losses occur. Also, as already pointed out, a seasonal variation is to be expected in regard to the carbohydrate content. All of the plants were good sources of alginic acid, laminarin, and mannitol; although they are more difficult to gather than other species yielding comparable quantities of these constituents.

In the antibiotic survey, only one of the plants, *Cymathaere triplicata*, gave positive results. Extractions of this plant consistently gave indications of antibiotic properties, which qualitatively resembled those given by penicillin G in a solution of 100 units per cc., when tested in the same manner as the plant. The fact that very little antibiotic potency is shown by the other plants is a point in favor of the conclusion that *Cymathaere triplicata* has definite antibiotic properties, since it tends to rule out the influence of extraneous factors which might be common to all such types of plants. In case of continued positive results with this plant, it would form the basis of a good chemical problem embodying the attempted isolation and characterization of the active substance or substances.

TABLE V.—RESULTS OF ANTIBIOTIC SURVEY OF THE KELPS

Kelp	Whole Plant	Ammonium Sulfate Extract	$NaHCO_3$ Extract, Unconcentrated	$NaHCO_3$ Concentrated, Double Strength	Method of Pratt, Daniels, *et al.*
Hedophyllum sessile	Slight inhibition	Negative	Negative	Negative	Negative
Egregia Menziesii	Negative	Negative	Negative	Negative	Negative
Cymathaere triplicata	Negative	Positive 18-mm. diameter zone	Slight inhibition	Positive 16-mm. diameter zone	Negative

In investigating the vitamin content of these kelps, only the water-soluble vitamins were studied, since it was apparent from the absence of sufficient lipoids in these plants that useful concentrations of fat-soluble vitamins were not present. It is evident, however, that the plants are a rich source of vitamin B_1, niacin, and riboflavin. They also show a useful content of pantothenic acid, carotene, and vitamin C. The plants were collected in June and September and assayed in both the fresh and dried forms. No definite seasonal variation was indicated but it was shown that there was a high vitamin content at both seasons and that the vitamin content remained essentially high even after drying.

The results of the vitamin assays and ash determinations indicate the fact that these kelps would form an excellent adjunct to cattle food. Nor is it unreasonable to think that they might be valuable supplements in human diet if prepared in a palatable form. They might also be used as fertilizer though they are low in protein, but they would be valuable in rebuilding the mineral content of leached soils. These views are confirmed in similar studies by Lunde (11) on Norwegian marine brown algae. He found a lower content of thiamine in several plants studied and a higher content of ascorbic acid in fucus, than was found in the plants under consideration here.

Norris and associates (12) made a study of thiamine and ascorbic acid content of a large number of kelps collected from the same general area as those of this study. They found in general that these plants contained thiamine in amounts comparable to many fruits and vegetables. In their investigation of the vitamin C content of a number of kelps, *Egregia Menziesii* and *Hedophyllum sessile* were included. Their results are somewhat higher than those obtained in the present study. Their investigation did not include the determination of the thiamine content of these two plants.

SUMMARY

1. A partial proximate analysis and the mineral content of the plants have been made.

2. A determination was made of the three carbohydrates, alginic acid, laminarin, and mannitol.

3. The plant content of the water-soluble vitamins, carotene, ascorbic acid, thiamine, riboflavin, pantothenic acid, and niacin was determined.

4. In a survey of the plants, the presence of an antibiotic principle was indicated only in *Cymathaere triplicata*.

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Laboratory Assay of Clinically Effective Antiepileptic Drugs*,†

By EWART A. SWINYARD

Clinically useful antiepileptic drugs were assayed in rats for their ability to modify or prevent electrically or chemically induced seizures. The assays included modification of seizure pattern, ability to elevate normal and experimentally reduced electroshock threshold for minimal seizures, and antagonism to Metrazol. Toxic doses (TD_{50}) and protective doses (PD_{50}) were determined graphically and the protective indices calculated (TD_{50}/PD_{50}).

SINCE the introduction of diphenylhydantoin for the treatment of grand mal and psychomotor epilepsy, a large number of compounds have been tested for possible antiepileptic activity (1–5). Most of these drugs have been evaluatcd in laboratory animals either by their ability to raise the normal electroshock threshold or to prevent Metrazol and other chemically induced convulsions (1, 3, 5, 6). In many instances the experimental evaluation of antiepileptic activity has been made solely on the basis of a single criterion (3, 5, 6). It has become apparent that protective action against different epileptic manifestations (e.g., grand mal, petit mal) can be embodied in different drugs (7). Experimentally it has been shown that not all antiepileptic drugs have the ability to increase normal electroshock threshold, although anticonvulsant activity can be demonstrated by other electrical and chemical tests (8–12), and that not all antiepileptic drugs have protective value against Metrazol convulsions. It should be emphasized that up to the present time there is no single laboratory test available which in itself will definitively demonstrate the presence or absence of antiepileptic activity of a drug. For this reason it was thought advisable to integrate the most effective techniques known at present into a comprehensive routine assay procedure in such manner as to provide a means for rapidly screening and differentiating large series of new substances. Brief reports of the tests employed have been previously published (11–14).

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METHODS

From a considerable experience in screening large numbers of compounds for antiepileptic activity in various species, it has been found that the rat is the most convenient laboratory animal (11, 15). Sprague-Dawley male albino rats of uniform weight were used because they are easy to handle and respond uniformly. They were maintained on an adequate diet (Purina fox chow) and food was removed only during the actual assay.

For assays based on electrically induced convulsions, Spiegel (16) corneal electrodes were used and 60-cycle alternating current was delivered by means of an apparatus designed by Dr. L. A. Woodbury; the current was independent of the external resistance. Stimulus duration was 0.2 second. In all electroshock tests, the animal was restrained only by hand and released at the moment of stimulation in order to permit observation of the seizure throughout its entire course.

Whenever possible the drugs under investigation (sodium Mebaral, sodium phenobarbital, sodium diphenylhydantoin, sodium 5-thienyl-5-phenylhydantoin and trimethadione) were administered subcutaneously in aqueous solution. Compounds insoluble in water (Epidon, Phenurone) were suspended in 10% acacia solution and given orally. Drugs which were found to be poorly absorbed from the gastrointestinal tract (Mesantoin) or immiscible with water (Paradione) were dissolved in propylene glycol and given intraperitoneally. When propylene glycol was used, the concentration of drug was adjusted so that the maximum total dose of solvent administered did not exceed 1.5 cc. per kilogram. All subcutaneous injections were made into a loose fold of skin in the midline of the back. Shoulder and pelvic girdle areas were avoided in order to eliminate sequelae of the injection which might erroneously be interpreted as neuromuscular deficit.

The various anticonvulsant tests were made at the previously determined time of peak drug effect, i.e., forty-five to sixty minutes for oxazolidine-2,4-diones and barbiturates, four to five hours for substituted 5-phenyl hydantoins, and three hours for Phenurone

General Procedure

The four standard tests used routinely are described below. Three of these tests are electrical and one is chemical. In addition, a toxicity determination is made. The tests employed are:

1. Maximal Electroshock Seizure (M.E.S.) Test.
2. Normal Electroshock Threshold (N.E.T.) Test.
3. Hydration Electroshock Threshold (H.E.T.) Test.
4. Metrazol Test.

The concentration of drug required to produce the desired effect in each test in 50% of experimental

animals (ED_{50}) is determined in two stages: (*a*) A series of five or six animals is given doses of the drugs to be tested in successive twofold steps until an effective dose range has been reached, i.e., the lowest dose which provides protection in each test and the highest dose which gives no protection. (*b*) Groups of 4 to 6 animals are then used for each of a series of dosages within the indicated effective range, and the percentage of animals protected in each group determined. The percentages are converted to probit values and plotted against log concentration. The effective dose (ED_{50}) and its standard error are determined graphically by the method of Miller and Tainter (17). Finally, the dose which is toxic to 50% of animals (TD_{50}) is divided in turn by each of the ED_{50} values for the four anticonvulsant tests, to give four protective indices.

To calculate the standard error of each protective index, the two determined standard errors (of the toxic and effective doses) were converted to fractional parts of their respective values, and the root mean square of these fractional values was then multiplied by the protective index. The standard errors so obtained are first approximations and should not be used for critical comparisons of the significance of the results.

Standard Tests

In each of the following tests, care is exercised to exclude animals which have not fully recovered from the effects of previously administered drugs. The tests may be combined in various ways in order to conserve animals; for example, toxicity data may be obtained from the same animals used in the M.E.S., N.E.T., or Metrazol test.

(1) **Maximal Electroshock Seizure (M.E.S.) Test.**—Animals are given a single supramaximal shock with a current intensity of 150 mA., which is approximately 5 times threshold. All control animals should exhibit a definite extension of the hind legs during the tonic phase of the seizure, and aberrant animals not manifesting tonic extension are not employed for this test. Abolition of this extensor component after drug treatment is taken as the end point of this test (11–15, 18).

(2) **Normal Electroshock Threshold (N.E.T.) Test.**—For initial determination of control threshold for minimal electroshock seizures, shocks of 0.2 second duration are given at intervals of six to eight hours, with small (3%) increments or decrements in current intensity until a "minimal" seizure occurs. In rats, minimal seizures are defined as those which consist of ten or more seconds of facial clonus without loss of righting reflexes. With this criterion the normal threshold for minimal electroshock seizures (159 rats) varies between 20 and 36 mA. (average 28 ± 0.29 mA.), and a change of 5% in threshold can be detected without difficulty. For any one animal, the threshold is quite constant. However, it does vary from rat to rat with age, nutritional state, environmental and body temperature, etc. (19, 20). For determination of drug action, a single shock having a current intensity 20% above control threshold is used. Absence of even a minimal seizure is taken as the end point of this test.

(3) **Hydration Electroshock Threshold (H.E.T.) Test.**—For control determinations, the minimal electroshock seizure threshold is determined as before. Four hours before the anticipated time of peak drug effect, each animal is injected intraperitoneally with 10 cc. of freshly prepared isomolar (5.5%) glucose solution per 100 Gm. body weight. It has previously been shown (9) for a large series of animals that four hours after injecting the glucose solution, the electroshock threshold is reduced to 44 ± 1.26% of its initial value. To test drug effectiveness in preventing this degree of reduction in threshold, a single test shock is given having a current intensity 66% of the normal electroshock seizure threshold for each animal. This current intensity represents a 50% increase above the experimentally lowered hydration threshold. Absence of even a minimal seizure is taken as the end point of this test.

(4) **Metrazol Test.**—It has previously been determined that a subcutaneous dose of 70 mg./Kg. of Metrazol (5% aqueous solution) will cause seizures in more than 97% of rats (CD_{97+}) (13). This amount is approximately twice the threshold dose for 50% of animals. Because of the high percentage of deaths with this dosage, control determinations are usually omitted. The test is carried out by making the Metrazol injection approximately ten minutes before anticipated time of peak anticonvulsant drug action and observing the animals during the following sixty minutes for the occurrence of seizures. A threshold convulsion is defined as one episode of clonic spasms which persists for at least a five-second period. Transient intermittent jerks or tremulousness are not considered as constituting a convulsion. Absence of even a threshold convulsion during an hour of observation is taken as the end point of this test.

Toxicity Determination.—The following 3 criteria were found most useful for disclosing minimal neurological deficit at the time of peak drug action.

Walking and Postural Test.—Neurological deficit is indicated by a circular or zig-zag gait, ataxia, abnormal spread of the feet, absence of postural reflexes, hyperactivity, stupor, catalepsy, etc.

Vertical Screen Test.—The normal rat will climb on a vertical screen (¼-in. mesh) and only occasionally lose its footing. Neurological deficit is indicated by a tendency to slip or inability to maintain its position on the screen.

Positional Sense Test.—If the hind leg of a normal rat is gently lowered over the edge of a table, it will be quickly lifted back to a normal position. Neurological deficit is indicated by inability rapidly to correct such an abnormal position.

Deficit disclosed by any of these three examinations for evidence of drug toxicity was taken as the end point. However, if other side effects consistently appeared at lower doses (hematuria, hyperexcitability, hyperpnea, etc.), these were taken as the end point.

RESULTS

In order to establish the validity of laboratory tests for predicting clinical specificity and potency of new anticonvulsant drugs, the clinically useful antiepileptics were assayed by the procedures outlined. The results are shown in Table 1 and Fig. 1.

Substituted 5-phenyl hydantoins (diphenylhydantoin, Mesantoin, 5-phenyl-5-thienylhydantoin) useful in grand mal are characterized in the laboratory by a high protective index by the maximal electroshock seizure test. A high protective index by the normal electroshock threshold or Metrazol test does

TABLE I.—PROTECTIVE INDICES OF CLINICALLY USEFUL ANTIEPILEPTIC DRUGS BY FOUR DIFFERENT ASSAY METHODS IN RATS

Drug	TD_{50}, Mg./Kg.	Protective Indices[a] Maximal Electroshock Seizure Pattern	Normal Electroshock Threshold	Hydration Electroshock Threshold	Metrazol Seizure Protection	Major Usefulness in Epilepsy
Mesantoin	50 ± 3.1	11.1 ± 0.71	0.69 ± 0.07	1.42 ± 0.25	0.91 ± 0.08	GM PsM
5-Thienyl, 5-phenyl hydantoin	75 ± 6.5	4.47 ± 0.42	0	0.53 ± 0.05	0	GM
Diphenyl-hydantoin	104 ± 8.0	2.36 ± 0.24	0	1.1 ± 0.09	0	GM PsM
Phenobarbital	22 ± 1.1	3.58 ± 0.26	1.75 ± 0.19	1.18 ± 0.12	0.85 ± 0.08	GM
Mebaral	38 ± 1.1	2.46 ± 0.08	1.28 ± 0.20	2.04 ± 0.16	2.51 ± 0.18	GM
Trimethadione	445 ± 27.7	1.23 ± 0.11	1.51 ± 0.17	1.17 ± 0.13	1.48 ± 0.13	PM
Paradione[b]	167 ± 11.5	0.7 ± 0.08	1.11 ± 0.10	1.05 ± 0.11	1.22 ± 0.14	PM
Epidon[c]	1200 ± 54.2	1.78 ± 0.25	1.00	1.00	0	GM
Phenurone[d]	730 ± 99.2	17.7 ± 2.60	18.7 ± 3.70	3.24 ± 0.72	3.65 ± 1.1	GM PsM PM

[a] For details of assay procedures and end points employed, see text. GM, grand mal; PsM, psychomotor; PM, petit mal. ±S.E.
[b] 3,5-Dimethyl-5-ethyl oxazolidine-2,4-dione.
[c] 5,5-Diphenyl-oxazolidine-2,4-dione.
[d] Phenacetylurea.
Phenurone, Trimethadione, and Paradione were supplied by Abbott Laboratories; Mebaral by Sterling-Winthrop; Mesantoin by Sandoz Chemical Works; and 5-thienyl, 5-phenyl hydantoin by Eli Lilly & Co.

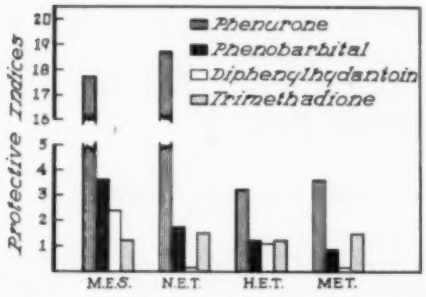

Fig. 1.—Profiles of Action of Anticonvulsant Drugs in Rats. "M.E.S.," maximal electroshock seizure test. "N.E.T.," normal electroshock threshold test. "H.E.T.," hydration electroshock threshold test. "MET.," Metrazol seizure test. Note the break in the ordinate scale of protective indices.

not appear to be an obligatory feature of this chemical nucleus. It is noteworthy that the substituted 5-phenyl hydantoins elevate the experimentally lowered threshold even though they fail to elevate significantly the normal electroshock threshold.

Barbiturates (phenobarbital, Mebaral) effective in grand mal show activity by all four assay procedures. They are particularly characterized by their ability to modify maximal seizure pattern and contrast sharply with the substituted 5-phenylhydantoins in their ability to elevate normal electroshock and Metrazol thresholds.

Oxazolidine 2,4-diones (trimethadione, Paradione), useful in petit mal, rank between the barbiturates and substituted 5-phenyl hydantoins by the normal and hydration electroshock threshold tests, and are a poor fourth by the maximal electroshock test. Metrazol antagonism is the most significant feature of these compounds and in this respect contrasts sharply with Epidon which is ineffective against Metrazol. It is of interest to note, that Epidon, also an oxazolidine 2,4-dione, resembles the substituted 5-phenyl hydantoins when assayed by these tests. Likewise, it is clinically more useful in grand mal than in petit mal.

Phenacetylurea (Phenurone) shows marked activity by all four tests. Indeed, Phenurone has the highest index by all four assay procedures of any compound yet tested. In this regard it is interesting to note that preliminary clinical trial indicates it is also effective against all three major seizure types (21).

DISCUSSION

Figure 1 shows the profile of action of four representative drugs, Phenurone, phenobarbital, diphenylhydantoin, and trimethadione, when assayed by the procedure discussed above. It is obvious that each test gives a different evaluation of anticonvulsant drug action, and that simple elevation of threshold is not alone adequate as a criterion of antiepileptic activity. For example, diphenylhydantoin neither raises threshold for minimal electroshock seizures nor prevents Metrazol convulsions. A potent and valuable antiepileptic like diphenylhydantoin could thus be missed if evaluated only by these tests. On the other hand, diphenylhydantoin elevates the experimentally lowered threshold for minimal seizures and markedly alters the pattern of maximal electroshock convulsions. The sharpest difference lies between the results of the maximal electroshock seizure and Metrazol tests. For this reason, and because they are rapid and easy to perform, these two tests are used for the preliminary screening of new drugs. However, no one laboratory test is sufficient to measure the potential anticonvulsant potencies of a new drug. Therefore, more promising compounds should be subjected to the remaining tests in order more definitely to demonstrate their anticonvulsant activity.

SUMMARY

Clinically useful antiepileptic drugs are assayed in rats for their ability to modify or prevent elec-

trically or chemically induced seizures. The assay methods include modification of seizure pattern, ability to elevate normal and experimentally reduced electroshock threshold for minimal seizures, and antagonism to Metrazol. Toxic doses (TD_{50}) and effective doses (ED_{50}) are then determined graphically and the protective indices calculated (TD_{50}/ED_{50}). The details of a routine assay procedure are described.

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Preliminary Report on the Volatile Oil of *Pycnanthemum Pilosum* Nutt.*,†

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A report on the physical and chemical constants of the oil is presented

INTRODUCTION

THE plant material for this study was obtained from the vicinity of Atlantic, Iowa, through the courtesy of Mr. Frank Pellett, who is interested in the cultivation of the plant for bee pasture. The 1945 crop was represented by 154 pounds of material harvested from an area of 432 square feet representing a yield of 15,500 pounds per acre. Distillation of the fresh plant material yielded 629 grams of deep yellow-colored oil which separated from the aqueous distillate, and cohobation yielded an additional 43 grams. The total yield of oil corresponded to 0.976 per cent of the fresh plant material.

The crops for 1946 and 1947 averaged 17,500 pounds per acre, with an oil yield of 0.95 per cent.

EXPERIMENTAL

Physical Properties.—All of the oil samples were yellow in color, but there was some variation in intensity, with the 1945 oil being the darkest. The odor was sweet and strong, suggesting the presence of pulegone. The physical constants, determined at 25°, are recorded in Table I.

Attempts to cause the oil to congeal or to yield a crystalline component were unsuccessful, even at a temperature of −60°.

Saponification Value.—The saponification values and an interpretation of them for the various oil samples are given in Table II.

No explanation is offered for the variations in the values obtained. The 1945 oil showed an unusually high solubility value, and originally it was felt that the cohobation process might have been conducted in a manner such that the recovery of water-soluble acids was high; however, the acid values of all of the oil samples were negligible.

To interpret the saponification value in terms of the alcohol in the original oil (Table II) it was assumed that the alcohol present is one of the isomeric forms of $C_{10}H_{19}OH$ and, to estimate the percentage of acetate ester, it was assumed that this alcohol occurs as the acetic acid ester (1).

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† Abstracted from theses submitted by Victoria Chilquist and Robert Gilmour for the degree of Bachelor of Science.

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TABLE I.—PHYSICAL CONSTANTS

	1945 Oil		1946 Oil	1947 Oil	
	Original	Cohobate		Original	Cohobate
Specific Gravity	0.9225	0.9483	0.9378	0.9233	0.9328
n_D^{25}	1.4690	1.4750	1.4795	1.4787	1.4708
$[\alpha]_D^{25}$	11.9	16.8	14.5	12.7	18.2
Solubility Value	560	658	...	...	...

TABLE II.—SAPONIFICATION VALUES

Sample	Sapon. Value	Per Cent Alcohol in Original Oil	Per Cent Acetate Ester
1945 original oil	38.0	10.90	13.44
1945 cohobated oil	82.4	24.34	28.99
1946 original oil	30.4	8.55	10.61
1947 original oil	15.5	4.37	5.48
1947 cohobated oil	12.8	3.60	4.52

Total Alcohol and Free Alcohol.—Duplicate samples of the original oil (1945) were acetylated in the usual manner, and the average saponification value of the acetylated oil was found to be 72.40. This value corresponds to 21.35% of total alcohol ($C_{10}H_{19}OH$) in the oil. Therefore, the calculated amount of free alcohol is 10.45%.

In like manner, the saponification value of the acetylated cohobated oil (1945) was found to be 98.00. This value corresponds to 27.3% of total alcohol ($C_{10}H_{19}OH$). Therefore, the calculated amount of free alcohol in this instance is 4.90%.

Acetyl Value.—Based on the saponification values of the acetylated samples, the acetyl value of the original oil (1945) was calculated to be 1.28, while that of the cohobated oil (1945) was 1.75.

Phenol Determination.—Carvacrol has been reported to occur in the volatile oil of *Pycnanthemum lanceolatum*, ranging from 7 to 58% (2). The phenol content of *P. pilosum* oil was determined, using 10-cc. samples, and found to be as follows:

1946 oil.....7.5%
1947 oil.....3.1%
1947 oil (cohobated).....4.6%

The free phenols were isolated from the alkaline aqueous solutions by acidification with hydrochloric acid and extraction with ether. After the ethereal solutions had been dried over anhydrous sodium sulfate and the solvent had been removed, the slight, brown, oily residues remaining represented the phenol fraction of the oils. All of the residues so obtained yielded a positive Flueckiger reaction (tan coating on NaOH pellet) and a brown color with ferric chloride solution, following the application of heat.

An attempt was made to prepare the phenyl urethane of the phenol, but without success. Even though the hydrocarbon solvent was dried over sodium, sufficient moisture was present to form the di-substituted urea which melted at 233°. Carvacrol phenyl urethane melts at 137°.

Ketone Determination.—The presence of pulegone was suggested by the odor of the various oils. Several methods were applied for the determination of the aldehyde and ketone content, but the results were not comparable, due to the pulegone being present. For example, the hydroxylamine methods (3) which were very slow, yielded high results because pulegone is capable of reacting with two moles of the reagent. Apparently, however, the reaction involving the addition of hydroxylamine at the point of unsaturation is not quantitative. The most consistent results were obtained by the sodium sulfite method. They are as follows:

1945 oil.....51%
1946 oil.....37%
1947 oil.....33%
1947 oil (cohobated).....21%

The ketone fraction was isolated from the assay solutions after the unreacted oil had been separated. These clarified aqueous solutions were made strongly alkaline with sodium hydroxide, and then steam distilled. The small quantities of oily ketone which separated were isolated and dried over anhydrous sodium sulfate. The physical constants for the ketone fractions are listed in Table III.

TABLE III.—PHYSICAL CONSTANTS FOR KETONE FRACTIONS

Sample	Specific Gravity	Refractive Index
1946 oil	0.9334	1.486
1947 oil	0.9319	1.488
1947 oil (cohobated)	0.9309	1.488

The similarity of these constants suggested the common identity of the water-insoluble ketone fractions, therefore they were combined and the optical rotation was measured. $[\alpha]_D^{25} = 22.25°$. The normal oxime melted at 124°. The physical constants for the ketone and its oxime are in agreement with those for pulegone.

Aqueous Cohobate.—In the initial distillation of the oil (1945), approximately 3 gallons of distillate were collected. Following the separation of the oil, the aqueous portion was cohobated. When the original volume had been concentrated by this process to 500 cc., the oily layer was removed, and the aqueous portion was further concentrated, but no more oil separated. The concentrate possessed a strong, honey-like odor, and this together with the high solubility value reported earlier indicated that there were present in the oil appreciable quantities of water-soluble components. The concentrate was examined for the presence of alcohols, aldehydes or ketones and acids.

A sample reacted positively with Fehling's and Tollen's reagents and with fuchsin-sulfite solution, indicating the presence of reducing substances like aldehydes and ketones. A 10-cc. portion of the

solution was neutralized by 0.437 cc. of 0.1 *N* sodium hydroxide solution. Calculated as acetic acid, this corresponds to 0.026%, indicating a negligible acid content. The Schotten-Baumann reaction, when applied to the sample, yielded a few drops of oily benzoyl ester, b. p. 135°, $n_D^{30} = 1.4670$. The yield was too small for additional data.

SUMMARY

In a preliminary report, the physical and chemical constants for the volatile oil of *Pycnanthemum pilosum* are given for samples obtained during three successive years. The presence of phenols is indicated, and pulegone was isolated and identified. The aqueous cohobate was shown to contain an alcohol and an aldehyde or ketone.

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A Comparative Study of the Effect of Various Solutions on the Phosphorus Depletion of the Rat Tooth Using Radioactive Phosphorus*,†

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Rat incisors become radioactive when labeled phosphorus is injected intraperitoneally. A new technique using radioactive isotopes as tracer elements in the study of the effect of various solutions on the teeth is described. Applications of the method are stressed.

ONE OF OUR greatest problems of the present day is the care and protection of teeth. Poor teeth represent the most common disorder of civilized man; more than 90 per cent of all persons have tooth decay or other diseases of the mouth at some time or another during their lives (1). At a time when medical science has triumphed over many infectious diseases, tooth decay presents problems which have baffled the dental and medical professions. One of the main problems of commercial concerns is the difficulty which they encounter in testing the action and effect of their products on the teeth. It is extremely difficult to test these preparations using the techniques described in the literature.

Perhaps the most important observation was made by Cox (2) in a review article in which he stated, "investigators, working with teeth, have no satisfactory method for reporting data." Some methods reported in the literature for determining the action of solutions on teeth consist of data based on the loss of weight of a tooth after immersion in solutions (3), examination of teeth with a reflecting microscope (4, 5), examination of photomicrographs of ground longitudinal sections (6), and by scratching the tooth with a sharp-pointed instrument by which teeth showing softening were classified as decalcified (7). These methods are reported to be somewhat unsatisfactory.

In recent years the use of radioactive tracers in animal and human metabolism has come into prominence. Greenburg (8) reported that Chievitz and Hevesy in some of their first experiments found radioactive phosphorus in the teeth of animals after administration. This suggests the possibility of the use of radioactive isotopes in the study of teeth.

Following the early work of Hevesy in which he showed that radioactive phosphorus was deposited in teeth, various workers have used this tracer element in studying the metabolism of laboratory animals. Manly and Bale (9) using P^{32} found rats' incisors showed a 2.0–3.5 per cent uptake of P^{32} in four hours and 8 per cent uptake

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in twenty days. Hevesy and Armstrong (10) reported that *in vitro* experiments showed the P^{32} acquired by enamel *in vivo* is apparently not delivered from saliva.

Hurley (11) showed that the most economical and efficient method for causing rat teeth of a heterogeneous group of rats to become radioactive with radioactive phosphorus to such an extent that the teeth contain at least 35 counts per minute per mg. (as measured 1.3 cm. from an Eck and Krebs counter tube) is to inject the rats intraperitoneally with a radioactive potassium phosphate solution containing between five and seven million counts per minute per cc. and remove the teeth twenty hours after injection. There are various other workers who have shown the uptake of labeled phosphorus in the teeth of various animals (12–21).

The literature offers no effective quantitative method for the demonstration of the effects of various solutions on the mineral depletion of teeth. One of the purposes of this work was to develop a method using a radioactive isotope as a tracer element by which the mineral depletion of teeth could be determined. Such a technique is described in the following experimental section and should prove to be of considerable value in the study of normal and abnormal metabolism of teeth where a precise method capable of detecting small differences in mineral content of the teeth is desirable.

EXPERIMENTAL

Before carrying out experiments involving the up take and depletion of phosphorus in rats' teeth using radioactive phosphorus as a tracer element, it is desirable to know the mode of entry of phosphorus into the tooth. If considerable amounts of labeled phosphorus are adsorbed on the tooth from the saliva the interpretation of results would be quite different than if labeled phosphorus enters the tooth through the blood stream.

An experiment was carried out to determine if phosphorus entered the rat tooth through the saliva. The upper teeth were covered with a metal cap and the lower teeth were left exposed to the saliva. Since it was necessary to have the teeth covered for a period of twenty hours, a "muzzle" was designed from discarded X-ray film to prevent the rat from removing the metal cap. Two holes were made in the film for the rat's front legs, and the rat was placed in the muzzle. The film was brought up over the rat's head and stapled close to the body. This is a convenient, rapid method for restricting the animal movements as desired, and may be used for other experiments where restriction of the animal is necessary.

The labeled phosphorus uptake was the same in the capped and uncapped teeth indicating that no phosphorus was taken up from the saliva.

The effect of various solutions on the labeled phosphorus content of rats' incisors was carried out on a qualitative basis as a preliminary to quantitative determinations. Radioactive teeth extracted from albino rats were subjected to various solutions and the activity of the solutions determined, indicating the extent of phosphorus depletion. The results obtained are summarized in Table I.

The procedure for the quantitative determinations of the labeled phosphorus depletion of rats' teeth when exposed to various solutions is described in detail as follows:

A known quantity of radioactive potassium biphosphate (KH_2PO_4), weighed accurately, was dissolved in 100 cc. of distilled water. This solution was standardized and the time and date of standardization noted.

Albino rats of medium size were injected intraperitoneally with that amount of KH_2PO_4 which had an activity of approximately 5,000,000 counts/min./cc. The rats were sacrificed after twenty hours by the use of chloroform. They were decapitated with a pair of scissors and the heads boiled in tap water for ten to fifteen minutes. The heads were removed from the boiling water and the incisors extracted. The teeth from each rat were placed in a separate beaker and 10 ml. of 0.05% KOH was added and the solution brought to a boil to clean and remove all extraneous material. The teeth were removed immediately, washed with tap water until free of KOH (about 5 washings), washed once with distilled water, and placed on a filter paper to air dry.

One of the four incisors from each rat was used as a standard. This tooth was ground to a fine powder in a Wedgwood mortar and 5.0 mg. of the powdered tooth was placed in a Kimbleware cup. One drop of 20% sulfuric acid was added and the contents of the cup charred in a hot-air oven (145°) after which the contents of the kimbleware cup were ashed in a muffle furnace at 500–550° and checked for radioactivity by the Geiger-Müller Counter.

The other three incisors were treated in the following manner before being placed in the test solution. Between one-eighth and one-fourth inch, depending on the size of the tooth, was removed from the root end of each tooth by using an electric dental saw. The root end of the tooth was then buffed until the teeth were all the same weight when checked on a Torsion balance. The ends of the teeth were covered with shellac; care was taken that no shellac was placed on the body of the tooth. After the shellac dried, each tooth was placed in the test solution and left for seventeen hours. Six-inch Pyrex test tubes were used to hold the solution which was held in a constant temperature bath (37.5°). The test tubes were stoppered to prevent evaporation. After seventeen hours the teeth were removed from the test solution. The test solution was evaporated in kimbleware cups to dryness by placing the cups in a hot-air oven. One drop of 20% sulfuric acid was added and the contents of the cups ashed in a muffle furnace at 500–550°. The ash was then checked for radioactivity by the Geiger-Müller Counter.

In a recent article Wernimont (22) reviewed the use of control charts. A control chart may be described as a graphic presentation of test data in such a manner that the variability of all the results is compared with the average variability within (arbitrary) small groups of test results. The "control"

TABLE I.—SUMMARY OF DATA OF QUALITATIVE DETERMINATIONS OF THE PHOSPHORUS DEPLETION OF RATS' TEETH WHEN EXPOSED TO VARIOUS SOLUTIONS

Solution Tested	pH	Number of Teeth Used[a]	Average Activity per 5-Cc. Sample in Counts/Min.
Product A[b]	2.95	60	18.7
Product A	2.95	11	12.2
(Teeth previously exposed to Product A for ten hours)			
Product A (colorless)	2.95	16	41.8
Tap water	6.60	14	35.3
Normal saline solution (0.9% NaCl)	6.90	23	60.1
Saccharin 0.036 Triple-distilled water, *q. s.* 100 cc.	2.75	23	254.8
Saccharin 0.036 Zinc chloride 0.219 Triple-distilled water, *q. s.* 100 cc.	2.95	23	203.9
Saccharin 0.036 Zinc chloride 0.219 Absolute alcohol 5.0 Triple-distilled water, *q. s.* 100 cc.	2.65	23	53.2
Buffer Solution U. S. P.	1.15	4	3292.5
Buffer Solution U. S. P.	1.95	5	961.5
Buffer Solution U. S. P.	3.00	4	840.2
Buffer Solution U. S. P.	4.05	5	617.0
Buffer Solution U. S. P.	4.85	5	465.0
Buffer Solution U. S. P.	5.95	5	173.0
Buffer Solution U. S. P.	7.00	6	68.8
Buffer Solution U. S. P.	7.80	6	53.9
Buffer Solution U. S. P.	8.75	1	35.5
Buffer Solution U. S. P.	9.60	1	30.5
Zinc chloride 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.* 100 cc.	2.90	5	442.6
Zinc chloride 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.* 100 cc.	2.90	5	521.6
(Teeth previously exposed to solution for ten hours)			
Saccharin 0.036 U. S. P. Buffer (*p*H 3.00), *q. s.* 100 cc.	2.90	5	300.6
Saccharin 0.036 U. S. P. Buffer (*p*H 3.00), *q. s.* 100 cc.	2.90	5	291.0
(Teeth previously exposed to solution for ten hours)			
Saccharin 0.036 Zinc chloride 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.* 100 cc.	2.95	5	488.8
Saccharin 0.036 Zinc chloride 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.* 100 cc.	2.95	5	561.0
(Teeth previously exposed to solution for ten hours)			
Absolute alcohol 5.0 cc. Triple-distilled water, *q. s.* 100 cc. Absolute alcohol 5.0 U. S. P. Buffer (*p*H 1.95), *q. s.* 100 cc.	2.20	7	441.3
Triple-distilled water	6.20	47	76.0
Triple-distilled water	6.20	7	45.0
(Teeth previously exposed to solution for ten hours)			

[a] The teeth were placed in each solution for a period of ten hours.
[b] Commercial mouthwash.

shown by the control charts formulated from the results obtained by this technique was excellent taking into consideration the variability of a biological animal such as the rat.

The qualitative results indicate that the *p*H of a solution may have considerable effect upon the phosphorus depletion of the teeth; however, the quantitative results indicate that the *p*H is not the only factor that effects the removal of phosphorus. Triple-distilled water, having a *p*H of 6.15, removed an average of 3.16%, whereas tap water with a *p*H of 7.05 removed 0.98%, and a commercial mouthwash with a *p*H of 2.95 removed only 0.92%. Therefore, factors other than *p*H have an effect upon phosphorus depletion. It was found in these experiments that the combination of absolute alcohol along with deionized water had an effect in preventing phosphorus depletion in rats' teeth *in vitro*.

The commercial mouthwashes tested removed in each case somewhat less phosphorus than does triple-distilled water and in most cases slightly more than tap water.

It was of considerable interest to test a cola beverage for phosphorus depletion by this technique since a large amount of soft drinks are consumed each year. The particular product tested removed approximately twice as much phosphorus as triple-distilled water and approximately five to seven times as much as the commercial mouthwashes tested.

Since candies have been reported to be detrimental to the teeth and since most candies contain glucose, it was of interest to determine the phosphorus deple-

TABLE II.—SUMMARY OF DATA OF QUANTITATIVE DETERMINATION ON THE PHOSPHORUS DEPLETION OF RATS' TEETH WHEN EXPOSED TO VARIOUS SOLUTIONS

Solution Tested	pH	Number Teeth Used	Per Cent Labeled P Removed	Standard Error
Tap water	7.05	54	0.98	0.033
Triple-distilled water	6.15	72	3.16	0.135
Zinc chloride..... 0.219				
Saccharin..... 0.036				
Triple distilled water, *q. s.*.....100 cc.	3.05	66	3.62	0.145
Zinc chloride..... 0.219				
Saccharin..... 0.036				
Absolute alcohol..... 5.0				
Triple-distilled water, *q. s.*.....100 cc.	2.95	72	2.16	0.071
Zinc chloride..... 0.219				
Saccharin..... 0.036				
Absolute alcohol..... 5.0				
Deionized water, *q. s.*.....100 cc. (as used in preparation of Product A)	2.91	72	0.83	0.039
Product A[a]	2.95	72	0.92	0.052
Product B[a]	3.55	72	1.51	0.079
Product C[a]	4.65	72	1.14	0.086
Product D[a]	3.25	72	2.69	0.091
Product E[a]	7.25	69	2.96	0.271
Antiseptic Solution N. F.	5.35	66	0.97	0.085
Cola Beverage	2.50	63	6.28	0.147
Glucose 5% (distilled water solution)	4.90	63	3.88	0.085
Lemon juice	2.29	57	71.74	1.880
Grapefruit juice	3.25	60	38.97	0.967

[a] Commercial mouth washes.

tion of teeth in a 5% glucose solution in distilled water. The result compared closely to that obtained with triple-distilled water alone.

Approximately 70% of the labeled phosphorus in rats' incisors was removed by the action of lemon juice. This compares with the clinical findings of reports on the demineralization effects of lemon juice on human teeth (23). Grapefruit juice was found to remove approximately 40% of the labeled phosphorus from rats' incisors.

It must be emphasized that the results obtained in this work do not indicate the amount of phosphorus removed under normal conditions of usage of these substances *in vivo*, but are relative values based on *in vitro* tests which indicate that certain substances have greater ability to cause phosphorus depletion as compared to other substances under the conditions of the experiment.

In order to obtain significant results in these experiments, a total of approximately 2500 rat teeth representing approximately 1000 rats were used. Approximately four hundred and twenty hours were required to determine the radioactivity of various samples using the Geiger-Müller Counter.

SUMMARY

When labeled phosphorus is injected intraperitoneally into rats the rat incisor becomes radioactive. It has been shown that none of the phosphorus is adsorbed on or absorbed through the normal tooth enamel from the saliva. The labeled uptake of phosphorus was the same in capped and uncapped teeth.

A new convenient rapid technique for restricting the animal movements, using discarded X-ray film, has been described.

A new technique using radioactive isotopes as tracer elements in the study of the effect of various solutions on the teeth has been described. This technique has wide applications and has been used in this work to study the effect of certain mouthwashes, beverages, fruit juices, triple-distilled water, and tap water on the phosphorus depletion of rat teeth. Although the phosphorus depletion of teeth is not as important as the calcium depletion, the results obtained with phosphorus are perhaps indicative of calcium depletion since, although not entirely proved, most investigators feel that the calcium and phosphorus are in chemical combination in the tooth. The technique, however, may be used equally as well with the study of calcium or strontium depletion of the teeth since radioactive strontium and calcium are now available.

Experimentation is being continued on the problems involved with the effect of various solutions on mineral depletion of teeth with a view to improve the techniques involved and to obtain further information concerning the mechanism of depletion.

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Studies on Pharmaceutical Powders and the State of Subdivision. I. The Application of Low-Temperature Nitrogen Adsorption Isotherms to the Determination of Surface Areas*,†

By J. V. SWINTOSKY,‡ S. RIEGELMAN,§ T. HIGUCHI and L. W. BUSSE

Although it has been shown qualitatively that the pharmacological effects of sparingly soluble drugs are strongly influenced by the fineness of these pharmaceuticals, very little in the nature of quantitative study of this phenomenon has been attempted. A project has been initiated to correlate pharmacologic effects of slightly soluble pharmaceutical powders with their specific surface areas. In the present paper, which is the first of a series proposed in this program, the practicability of the nitrogen adsorption method for the determination of specific surface areas of pharmaceutical powders is shown. The significance of particle dimensions as calculated by these results is also discussed. Data are presented illustrating the application of the method to representative samples of titanium dioxide, zinc oxide, and barium sulfate.

THE PRESENT INQUIRY into the state of subdivision of pharmaceutical powders was initiated for a number of reasons. Precise standards have not been established for particle size and surface areas of pharmaceutical powders and as a consequence the size and surface relationships of presently available powders are not well known. There appear to be significant relationships between surface characteristics of powders and their physiological activities, yet only few quantitative correlations of these properties have been made. Also in the technology of pharmaceuticals surface and size characteristics obviously play an important role; however, little emphasis has ever been placed on uniformity and precise particle size values for this purpose.

Surface properties, especially specific surface areas,[1] are particularly important in slightly soluble substances since they are factors in solution rate, solubility, and absorption. It is then reasonable to assume that therapeutic response and antiseptic activity are closely related to the surface characteristic of those drugs which fall into the category of being slightly soluble. Some evidence to this effect has already been presented by a number of workers during the course of investigations on such substances as sulfadiazine (1), sulfathiazole (2–4), sulfur (5–8), phenolphthalein (9), mercury compounds (10, 11), and certain insecticides (12–18). From the standpoint of pharmaceutical technology, the role of particle dimensions in suspensions, emulsions, lotions, magmas, ointments, and similar preparations is important in achieving stability and elegance preparations of these types.

Comprehensive studies on the physical characteristics of pharmaceutical powders would then appear to serve a useful purpose. These studies should employ sufficiently precise analytical methods so that the data obtained could be applied to therapeutic and pharmaceutic investigations where the results could be interpreted quan-

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[1] The specific surface area of a powder is the sum of the surface areas of all the particles in a gram of the powder. It is an intensive property of the powder similar to density, specific heat capacity, etc.

titatively according to well-known physical and chemical laws.

A number of methods for the determination of size and surface characteristics of powders are available. The purpose to which such data are to apply would govern to some extent the choice of analytical procedure to be followed. Some of the more important methods of powder analysis are listed below (21).

1. Sieve Analysis
2. Microscopic Analysis
   - A. Simple Microscopic Methods
   - B. Ultramicroscopic Methods
   - C. Electron Microscopic Methods
3. Sedimentation Analysis
   - A. Increment Methods
     - (*a*) Pipette Method
     - (*b*) Photographic Method
     - (*c*) Pressure Method
     - (*d*) Hydrometer Method
   - B. Cumulative Methods
     - (*a*) Balance Methods
     - (*b*) Pressure Methods
4. Centrifugal Analysis
   - A. Ordinary Centrifugal Methods
   - B. Supercentrifugal Methods
   - C. Ultracentrifugal Methods
5. Elutriation Analysis
   - A. Air Elutriator Methods
   - B. Liquid Elutriator Methods
6. Turbidimetric Analysis
   - A. Gross Methods
   - B. Size Distribution Methods
7. Miscellaneous Methods of Analysis
   - A. Permeability Methods
   - B. Energy of Emersion Methods
   - C. Adsorption Methods

Criteria for selection of an analytical method and descriptions of such methods are available in standard references on this subject.

In the classification given above, permeability methods, energy of emersion methods, and adsorption methods constitute procedures for the absolute measurement of specific surface areas. An adaptation of the permeability method has seen application for this purpose (23) in the study of some pharmaceutical powders. The energy of emersion method requires very elaborate equipment and refined techniques not suitable for routine analysis of powders. The adsorption method was chosen for the present study because of its relative simplicity and proved utility.

This paper will be confined to an analysis of several pharmaceutical powders by gas adsorption. With this method it is possible to determine the type of van der Waal adsorption exhibited by each particular substance, the absolute surface areas may be ascertained, and the average particle size of nonporous powders may be calculated.

Gas adsorption as a method of determining the surface areas of finely divided porous or nonporous solid materials is not new. The method has, however, only recently found real practical utility in measurements of absolute surface areas as a result of the theoretical developments projected by Brunauer, Emmett, and Teller (19) (BET Method). It has seen a particularly prolific application industrially to the determination of surface areas of catalysts. A number of chemically different adsorbate gases have been employed successfully to this end.

For the project being carried out in this laboratory, nitrogen was chosen as the adsorbate since liquid nitrogen could be conveniently prepared for the cooling bath. Likewise, since some of the adsorbents being studied are organic in nature, it seemed desirable to abstain from using organic gases which might react with or be dissolved in the organic crystals. The method, theory, and application of gas adsorption for this purpose have been the subject of a number of journal and review articles (20, 21, 22), so that the present paper shall treat this aspect only briefly.

APPARATUS AND PROCEDURE

Adsorption Apparatus.—The adsorption equipment was mounted on a metal framework. This was then built on a low table set on casters, permitting free portability. The apparatus was of a standard design, with a few modifications, as shown in Fig. 1.

The sizes of the adsorption tubes were dictated by the nature of the adsorbent, smaller tubes being used where the surface area per unit volume of adsorbent was high. Two-millimeter capillary tubing was used to connect critical portions of the system in order to keep dead-space volumes at a minimum yet allowing for a reasonably rapid evacuation of gases during evacuation procedures. In the powders reported in this paper, adsorption analyses were preceded by evacuation of the samples for ten hours at 105° in order to remove adsorbed water and other extraneous substances from the surface of the adsorbents. The relative vapor pressures of the adsorbent samples ultimately determined the temperatures used in this particular operation. With substances of relatively high vapor pressures at this evacuation temperature the nature of the crystal surfaces would change appreciably by sublimation. The samples were evacuated at temperatures where the vapor pressure of the adsorbent remained well below 0.001 cm. of mercury. Space in the critical portion of the system, not occupied by either the sample or the calibrated burettes, was determined by employing pure helium since this gas is inert, is not adsorbed at room temperature, and obeys the laws of an ideal gas under the experimental conditions. This dead space, a, can be calculated by determining the pressure of a given amount of helium confined in the space plus variable known volumes, V', in the burettes. The equation would then take the form $P(V' + a) = \text{constant}$. The equation can be solved for a if several values of P are obtained for corresponding values of V'.

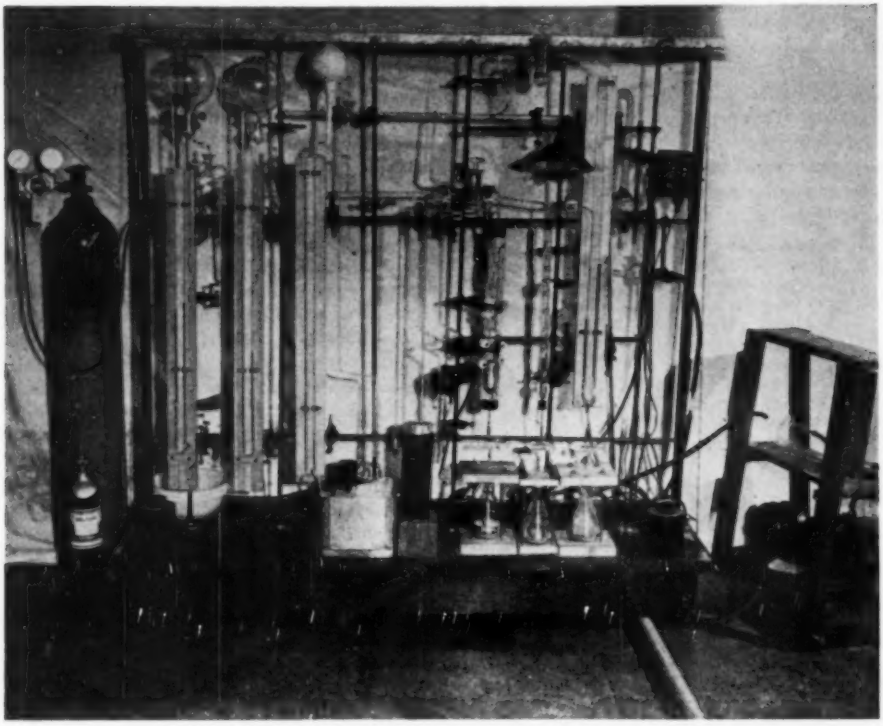

Fig. 1.—Adsorption equipment.

Since liquid nitrogen for cooling the sample tubes was not readily available, it was prepared by utilizing a liquid air condenser of simple design illustrated in Fig. 2.

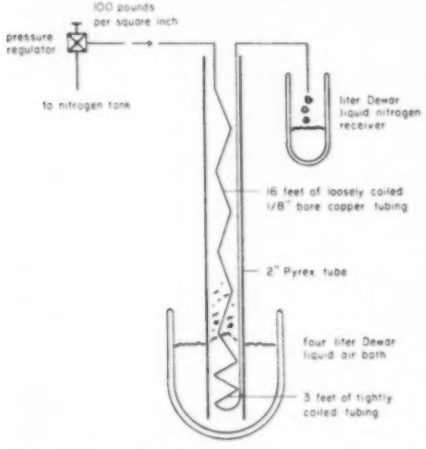
100 pounds per square inch
pressure regulator
to nitrogen tank
liter Dewar liquid nitrogen receiver
16 feet of loosely coiled 1/8" bore copper tubing
2" Pyrex tube
four liter Dewar liquid air bath
3 feet of tightly coiled tubing

Fig. 2.—Schematic diagram of liquid nitrogen condenser.

Precooled gaseous nitrogen at a pressure of 100 lb. per square inch was passed through the liquid air immersed coils and a liter of liquid nitrogen was obtained for every three liters of liquid air consumed. Greater efficiency could be attained if quantities larger than one liter of nitrogen were prepared at a single operation.

The temperature of the liquid nitrogen bath varied only slightly during successive runs. The values of this temperature and P_0, obtained by employing a nitrogen vapor pressure thermometer, indicated that P_0 ranged consistently between 69 and 72 cm. of mercury and ranged usually between 70 and 71 cm. of mercury so that a value of 77° K. was used regularly as the liquid nitrogen bath temperature.[1]

[1] By calculations involving the Clausius-Clapeyron equation

$$\log \frac{P_1}{P_2} = \frac{L}{2.303R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where

P_1 = 76 cm. of mercury; P_2 = value of P_0 observed on the vapor pressure thermometer; L = heat of vaporization of nitrogen, equal to 1323 calories per mole; R = the gas constant equal to 1.987 calories per degree per mole; T_1 = calculated absolute temperature for variable values of P_2; and T_2 = the equilibrium temperature of boiling liquid nitrogen in a nitrogen atmosphere at a pressure of 76 cm. of mercury, equal to 77.58° K.

It can be shown that T_1 is equal to 76.72° K. when P_0 (i.e., P_2 in the equation) is equal to 69 cm. of mercury and similarly T_1 is equal to 77.10° K. when P_0 is equal to 72 cm. of mercury. The deviation from an absolute value of 77° K. was thus so minimal that the latter value could be used consistently without introducing an error exceeding other experimental errors.

The adsorbate nitrogen used for these measurements was a specially pure grade of Linde tank gas which was used directly without further purification. The helium for dead-space measurements was a medical grade product supplied in cylinders by the Puritan Compressed Gas Corp. This gas was purified by conventional methods utilizing an activated charcoal train cooled to liquid nitrogen temperature.

Calculation.—The straight line isotherms obtained from the nitrogen adsorption data, were plotted in the relative pressure (P/P_0) range of 0.05 to 0.35 by substituting the appropriate experimental values in the *BET* equation

$$\frac{P}{V(P_0 - P)} = \frac{1}{V_m C} + \frac{(C-1)P}{V_m C P_0} \qquad (1)$$

where

V = volume of gas adsorbed per gram of adsorbent at pressure P at a temperature at which the vapor pressure of the liquefied gas is P_0,

V_m = volume of gas in cc. (converted to S. T. P.) required to form a monolayer on a gram of adsorbent,

C = a constant which is related exponentially to the difference between the heat of liquefaction and the heat of adsorption of the adsorbate.

From the slopes and intercepts, the respective values of V_m were obtained for the individual samples. The value of 16.2 Å.^{2} was used as the area occupied by a single close-packed nitrogen molecule adsorbed as a liquid monolayer film on the surface of the adsorbent. Thus one obtains the factor 4.35 which multiplied by the value of V_m yields directly the specific surface area in square meters per gram of adsorbent. In calculating the average particle size of the powders, the following equation was used:

$$d_m = \frac{6}{A\rho} \qquad (2)$$

where

d_m = surface average spherical diameter of particles in centimeters,

A = specific surface area in square centimeters of a gram of adsorbent, and

ρ = the density of the adsorbent material.

The specific surface areas of the following powders were determined in the apparatus described:

Titanium Dioxide.—The particular sample of titanium dioxide (anatase) studied was obtained from Professor W. D. Harkins of the University of Chicago. The material was from a lot which has been intensively studied by numerous investigators utilizing varied experimental approaches. For this sample Dr. Harkins obtained a value of 13.8 m^2/Gm. by using the energy of emersion technique. Several other workers employing the method of low-temperature nitrogen adsorption isotherms found an average value of 13.9 m^2/Gm.

Zinc Oxide.—Mallinckrodt U. S. P. grade zinc oxide was used without any pretreatment. The powder was dried over granular anhydrous magnesium perchlorate in a desiccator for at least a week, as were all other powders, prior to measurement.

Barium Sulfate.—Merck X-ray grade barium sulfate was used directly without further pretreatment aside from the drying operation.

RESULTS AND DISCUSSION

The results of the measurements on the three powders are shown in Figs. 3, 4, 5, and 6 and in

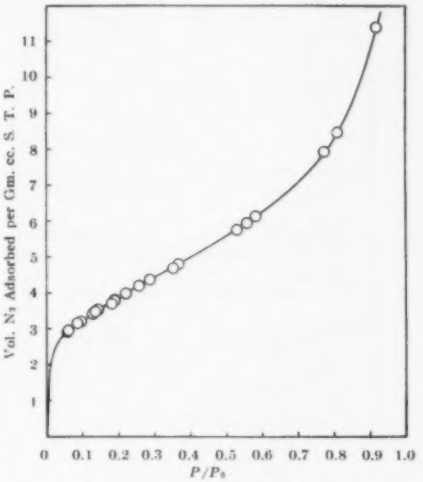

Fig. 3.—Adsorption of nitrogen on titanium dioxide (Sample No. 1A-6) at liquid nitrogen temperatures.

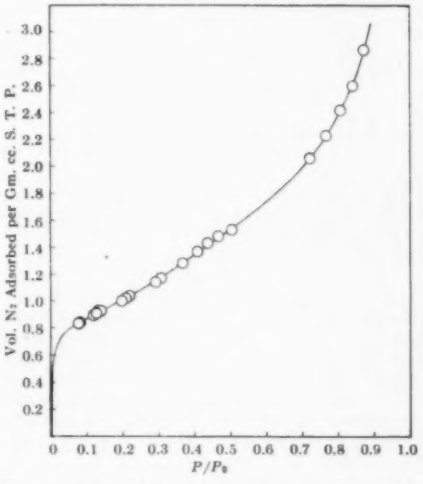

Fig. 4.—Adsorption of nitrogen on zinc oxide (Sample No. 2A-2) at liquid nitrogen temperatures.

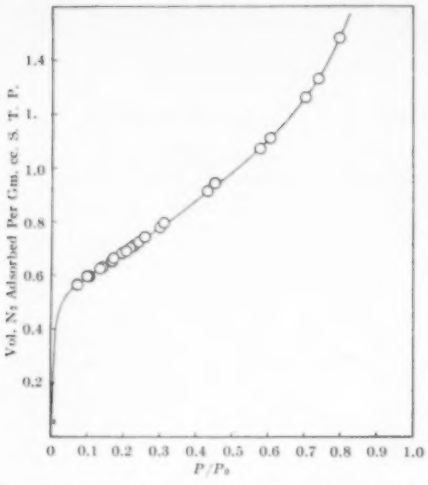

Fig. 5.—Adsorption of nitrogen on barium sulfate (Sample No. 3A-2) at liquid nitrogen temperatures.

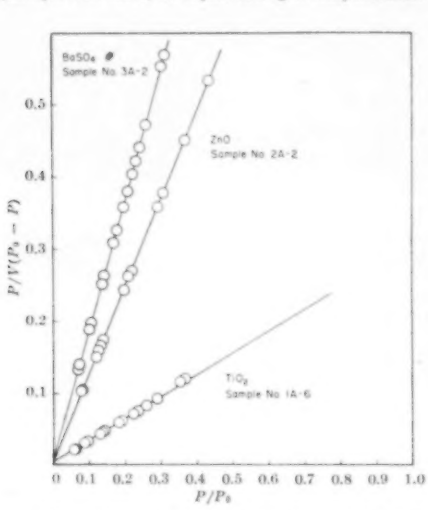

Fig. 6.—Adsorption data for nitrogen on titanium dioxide (Sample No. 1A-6), zinc oxide (Sample 2A-2), and barium sulfate (Sample No. 3A-2), plotted according to Eq. (1).

Table I. As shown in the tabulated data, numerous runs were made on the anatase sample in order to determine the relative and the absolute precision of the technique employed. The mean value of 13.7 m²/Gm. agrees satisfactorily with the results of other workers. For the sample of anatase, where ten separate values were obtained, the standard deviation of measurement calculated according to the equation

$$\sigma = \sqrt{\frac{\Sigma(x - x_0)^2}{n - 1}} \qquad (3)$$

TABLE I.—SURFACE AREA MEASUREMENTS ON SAMPLES OF TITANIUM DIOXIDE, ZINC OXIDE, AND BARIUM SULFATE BY NITROGEN ADSORPTION

Material	Sample Wt., Gm.	Analysis No.	Vm, cc.	Area, m²/Gm.
Titanium Dioxide[a] (Anatase), Sample No. 1A	2.247	1	3.20	13.9
	2.247	2	3.18	13.8
	3.530	3	3.26	14.2
	3.330	4	3.26	14.2
	3.310	5	3.07	13.4
	3.010	6	3.13	13.6
	3.000	7	3.10	13.5
	3.080	8	3.10	13.5
	3.150	9	3.10	13.5
	2.863	10	3.18	13.8[a]
Zinc Oxide,[b] Sample No. 2A	13.960	1	0.816	3.55
	4.050	2	0.816	3.55
	14.790	3	0.831	3.61
	13.087	4	0.811	3.52[b]
Barium Sulfate,[c] Sample No. 3A	20.086	1	0.549	2.39[c]
	17.060	2	0.557	2.42
	14.790	3	0.561	2.44

[a] Average area 13.7 m²/Gm.; density, 3.84; Diameter of average particles, 0.114 μ.
[b] Average area, 3.56 m²/Gm.; density, 5.47; diameter of average particle, 0.308 μ.
[c] Average area, 2.42 m²/Gm.; density, 4.50; diameter of average particles, 0.551 μ.

is 0.29 m²/Gm. The coefficient of variation for the titanium dioxide sample therefore is about 2%. It would thus appear that the accuracy and reproducibility of the method are quite adequate for the type of investigations contemplated.

Though the samples of zinc oxide and barium sulfate do not show any detectable difference in size by visual examination or by feeling them, the measurements would indicate a considerable relative difference from the titanium dioxide with respect to the particle size. This would indicate that such qualitative visual comparisons break down completely in this particle size range.

Hysteresis was not observed in the measurements on any of these powders.

SIGNIFICANCE OF THE RESULTS

If the powders under investigation consisted entirely of perfectly uniform spheres or cubes, the diameters listed in the table would conform very closely with the actual dimensions of the particles. However, the assumption is obviously too optimistic and the actual particles are more probably represented by a spectrum of sizes, the range varying for different materials and conditioned somewhat by the method of manufacture. The determined particle sizes in the case of these powders would be a surface mean particle diameter. Mathematically this could be represented as

$$d_{\text{surface average}} = \frac{\Sigma d_s^3}{\Sigma d_s^2} \qquad (4)$$

where the summation is over all of the particles. In comparison, the other types of averages take the following forms:

$$d_{\text{number average}} = \frac{\Sigma d_i}{n} \quad (5)$$

where n = total number of particles and

$$d_{\text{weight average}} = \frac{\Sigma d_i^4}{\Sigma d_i^3} \quad (6)$$

It is evident from these relationships that the surface average values would be somewhere intermediate between those obtained from number and weight averages.

Though the particle diameters determined by the gas adsorption method lose some of their significance because of the nonuniformity in size of the powder particles, the specific surface area—the area of the solid-air interface per gram of powder—is a real, definite value. Since specific surface area measurements permit ready calculation of the total surface area present in any given amount of powder, it should prove interesting to see whether the effective doses of certain slightly soluble drugs can be more directly correlated with the surface areas than with the weights of these substances. The solution rates and consequently the adsorption rates should be directly related to the exposed surface areas and therefore to the measured specific surface areas.

SUMMARY

The application of low-temperature nitrogen adsorption isotherms to the determination of specific surface areas and surface average diameters of pharmaceutical powders is illustrated in data for titanium dioxide, zinc oxide, and barium sulfate. Representative samples of these substances gave specific surface area values of 13.7 m²/Gm. for titanium dioxide, 3.56 m²/Gm. for zinc oxide, and 2.42 m²/Gm. for barium sulfate. The possible correlations of specific surface areas of slightly soluble medicinal powders with solution rates, adsorption rates, and therapeutic response are pointed out.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Glucose monocarbonate (any isomer)
L-Galactonolactone
Carboxymethoxylamine hemihydrochloride
3,5-Diiodotyrosine-*p*-nitrobenzyl ether
Hydroxypyruvic acid
2-Dimethylamino-1-chloropropane
3,4-Dichlorophenyl isocyanate
Sylvestrine
3,5-Diiodothyronine
Chavicine
12-Hydroxystearic acid
18-Hydroxystearic acid
p-Menthane
Vanillyl amine
d-Pantoyltauryl-*p*-chloroanilide
Vaccenic acid
Thymidine
3-Hydroxyanthranilic acid
3,7,3′,4′,5′-Pentahydroxyflavone
3,5,7,2′-Tetrahydroxyflavone

Effects of Surface-Active Agents on the Crystallinity of Carbowax Compounds*

By M. V. NADKARNI,† DONALD B. MEYERS† and LOUIS C. ZOPF‡

"Carbowax" 4000, a water-soluble waxlike compound, is a useful ingredient in washable ointment bases. The texture, unctuousness, and smoothness of the "Carbowax" containing base depend upon the degree of fineness of the crystals of "Carbowax." Several factors affect the size and form of crystals. Presence of synthetic wetting agents and emulsifiers in comparatively minute amounts has been demonstrated to have considerable effect on the size of the crystals. Several ionic and nonionic wetting agents in various concentrations have been studied. Photomicrographs have been made from the slides depicting crystallization from various combinations. Preliminary experiments show that some of these wetting agents are efficient in reducing crystal size.

IN RECENT YEARS, there has been an increasing tendency on the part of physicians to employ washable ointment bases in preference to the traditional oleaginous vehicles. In view of the existing confusion in the proper use of the term "washable," it may be well to point out that, as used here, it refers to the ease with which these bases and resulting ointments can be removed from the skin and clothing with water.

A great majority of these washable bases contain the water-soluble "Carbowax" compounds.[1] During the progress of our experiments on hundreds of washable ointment bases containing "Carbowax," it was observed that occasionally a rough, grainy ointment base of undesirable appearance could be changed into a smooth, uniform product by incorporation of a very small amount of a wetting or emulsifying agent. For pharmaceutical elegance, an ointment base must possess certain physical properties among which smooth texture and unctuousness are of prime importance.

A comparative microscopic study of slides prepared from bases made with and without the addition of surface-active agents in the formula revealed that those containing these agents showed improved appearance and uniformity. There were indications that this was due to the reduction in the size of the "Carbowax" crystals brought about by the presence of the surface-active agents. A study of the crystal behavior of "Carbowax" compounds and the effects of various factors affecting the size and form of crystals was, therefore, considered pertinent.

Very little work has been reported so far regarding the features of crystal structure of water-soluble "Carbowax" compounds. Paraffin wax and other petroleum waxes, on the other hand, have been extensively investigated and numerous reports have appeared describing their crystal structure and factors affecting it (2, 3, 6, 7, 12, 13).

Several factors other than the presence of surface-active agents seem to have an effect on crystal size of "Carbowaxes." As with other crystallizable compounds, it was observed that the rapidity of formation of crystals, the temperature to which the wax had been heated before allowing it to cool and crystallize, the molecular weight of the wax, the rate of cooling of the melted wax, the degree of purity of the material, and the amount of agitation during cooling of the melted wax are a few of the factors influencing the size of the crystals.

During this study, special care was taken to maintain external factors as uniform as was practicable. Because of the necessity to work with minute amounts of the "Carbowax" in order to obtain slides for microscopic study, the difficulty of rigidly controlling external conditions may be appreciated. The same lot of "Carbowax" 4000 was employed throughout this study. The temperature to which the wax was heated initially was the same in all cases, and the rate of cooling of the melted wax to room temperature was maintained as uniform as possible by enclosing the slide in a box insulated with board asbestos. Details of the procedure are given later.

Surface-active agents investigated consisted both of the ionizable and the nonionic type. Water was included because it is a constituent of

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‡ Professor of Pharmacy, College of Pharmacy, State University of Iowa.
[1] Products of the Carbide and Carbon Chemicals Corporation: They comprise a series of products of wax—like consistency having the general formula $HOCH_2—(CH_2—O—CH_2)_n—CH_2OH$. (The number following the word "Carbowax" refers to the approximate molecular weight of the wax.) Their physical properties vary with their molecular weights. The outstanding features of "Carbowax" compounds are their solubility in water and ability to form unctuous products when mixed with certain diluents, e.g., water, glycols, glycerin, etc., which makes them suited for use as a water-soluble ointment base.

all hydrous washable bases. The following substances were studied:

(1) Water
(2) Tergitol 4 (*a*)
(3) Tergitol 4 T
(4) Tergitol 7
(5) Tergitol 08
(6) Tween 80 (*b*)
(7) Tween 60
(8) Duponol C (*c*)
(9) Span 65 (*d*)
(10) Span 85
(11) Span 80
(12) Span 60
(13) Span 40
(14) Aerosol AY (*e*)
(15) Polyethyleneglycol 400 monostearate (*f*)
(16) Polyethyleneglycol 400 distearate
(17) Polyethyleneglycol 600 distearate
(18) Polyethyleneglycol 1000 monostearate
(19) Polyethyleneglycol 1540 distearate

(*a*) Tergitols: Products of Carbide and Carbon Chemicals Corporation. These are sodium salts and amine salts of higher secondary alkyl sulfates.

Tergitol 4: $C_4H_9—CH(C_2H_5)—C_2H_4—CH(SO_4Na)—CH_2—CH—(CH_3)_2$

Tergitol 4T: Amine salt of higher secondary alkyl sulfate (Triethanolamine derivative of 7-ethyl-2-methyl-4-undecanol)

Tergitol 7: $C_4H_9—CH(C_2H_5)—CH_2—CH(SO_4Na)—C_2H_4—CH—(C_2H_5)_2$

Tergitol 08: $C_4H_9—CH(C_2H_5)—CH_2—SO_4Na$

(*b*) Tweens: Atlas Powder Company
Tween 80: Polyoxyalkylene sorbitan monooleate
Tween 60: Polyoxyalkylene sorbitan monostearate

(*c*) Duponol C (Du Pont):
Sodium lauryl sulfate
$CH_3(CH_2)_{10}CH_2—O—SO_2—Na$

(*d*) Spans: Atlas Powder Company:
Span 85: Sorbitan trioleate
Span 80: Sorbitan monooleate
Span 60: Sorbitan monostearate
Span 40: Sorbitan monopalmitate

(*e*) Aerosol AY: Diamyl sodium sulfosuccinate (American Cyanamid Chemical Corporation)

(*f*) Polyethyleneglycol stearates: Carbide and Carbon Chemical Corporation and Kessler Chemical Company. These are mono- and distearates of the polyethylene glycols.

For observation of the crystal form of the pure waxes, a set of microscope slides were prepared. Another set was made to demonstrate the effects of addition of 10% to 40% of water to "Carbowax" 4000. The surface-active agents were employed in concentrations of 1% and 2 per cent. The aqueous phase of these respective mixtures was maintained at 10% and 20 per cent. The remainder of the mixture consisted of "Carbowax" 4000.

("Carbowax" 4000 was studied extensively because it has been demonstrated that, from the point of view of physical properties, "Carbowax" 4000 is the most suitable of the "Carbowaxes" so far examined for ointment formulation.)

PROCEDURE

"Carbowax" 4000 was heated to 70° in a small beaker on a water bath. The desired amount of the surface-active agent was weighed or measured and added to the molten wax with continuous stirring until a homogeneous mixture was obtained. A drop of the warm molten mixture at 70° was then removed with a small glass rod and carefully transferred to a microscope slide. A cover-slip was placed on the melted material without pressure and the slide immediately observed under the microscope.

Whenever water was employed in the formulation of the ointment base, the surface-active agent was separately dissolved in the water portion. This solution was heated to the same temperature as that of the wax before mixing. Further procedure was as above.

RESULTS

Observation of the gradual growth of "Carbowax" crystals under the microscope was an interesting and fascinating phenomenon. "Carbowax" compounds, without the addition of other ingredients, started crystallizing around a nucleus. Numerous dendrites radiated from this nucleus which was a random point. Similar growth from another nucleus coalesced with the first to form a ridge-like barrier. The crystals of pure "Carbowax" compounds were anisotropic; they showed a play of colors when observed under rotating Nicol's prisms. The crystals may be described generally as spherulitic plates.

SIZE OF CARBOWAX CRYSTALS

Wax	Average Size in mm.
Carbowax 1500	2.13×1.42
Carbowax 1540	1.2×0.78
Carbowax 4000	0.64×0.42
Carbowax 6000	0.28×0.21

Several slides were prepared for each concentration of the surface-active agents to insure that substantially the same type of crystals was formed each time under similar conditions.

Photomicrographs were made from each slide under polarized light since these gave a much improved definition and contrast in the final photographs (see pages 218 to 221). Both black and white and colored photographs were made. Magnification of the photomicrographs was the same in all cases, viz., about 400 diameters. The photographs are self explanatory with respect to the crystal size reduction. Only a few of the representative pictures are reproduced.

CONCLUSIONS

1. It appears that the physical properties of the waxes and their chemical constitution are an important factor in recrystallization, although

other associated substances mixed in deliberately or naturally also have considerable effect.

2. Upon slow cooling "Carbowax" compounds, *per se*, form large plate crystals from a nucleus. All members of the series show a marked similarity in crystal shape. They are described as plates with a nucleus and of spherulitic nature. The crystals display an anisotropic behavior revealed by a polarizing microscope.

3. The average size of the crystals formed from pure "Carbowaxes" under specified conditions diminishes as the molecular weight increases.

4. Addition of a diluent such as water reduces the average size of crystals but they still remain considerably larger than what may be termed desirable for an ointment base.

5. As a rule, crystals formed after the addition of surface-active agents lose their anisotropic nature and in some cases the form of the crystals changes from plate to the mal type. Mal type is characterized by lack of any definite recognizable form.

6. Surface-active agents have a definite effect on the size and form of "Carbowax" 4000 crystals. All those tested reduce the size, but their quantitative ability to reduce crystal size varies considerably.

7. The effects of the different surface-active agents and water may be briefly summarized:

(*a*) Water: Size of crystals is reduced but slightly by increasing the proportion of water. (Fig. 1-d.)

(*b*) Tergitols: Tergitol 4 in concentration of 1 per cent in presence of 9 per cent water reduces crystal size to a greater degree than any of the other surface-active agents studied. (Fig. 1-e.)

(*c*) Tweens: One per cent Tween 65 together with 9 per cent of water is superior to same concentration of Tween 80.

(*d*) Duponol C: Duponol is not very effective in reducing crystal size and does not give a uniform grain. (Fig. 1-g.)

(*e*) Spans: None of the spans are very effective in crystal size reduction. Plate-type crystals are changed to mal type (Figs. 1-h and i.)

(*f*) Aerosol AY is not greatly effective. (Fig. 1-j.)

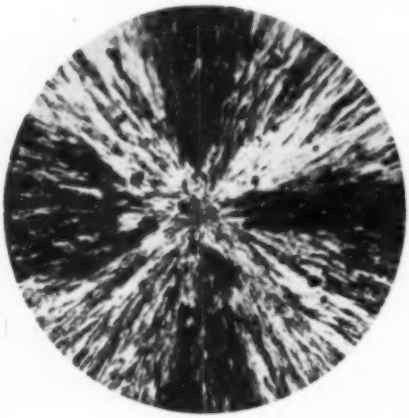
(*g*) Polyethylene Glycol Stearates: Polyethylene glycol 400 monostearate in concentration of 5 per cent and Polyethyleneglycol 400 distearate in concentrations of 5 and 10 per cent are effective crystal size reducers. Concentrations greater than 5 per cent of Polyethyleneglycol 400 monostearate in the former case and 10 per cent of Polyethyleneglycol 400 distearate showed no further reduction. (Figs. 1-l, m, o.)

(*h*) There appears to be an optimum concentration of any given surface-active agent which produces maximum effect on crystal size. Amounts in excess of this optimum are not only superfluous and without any further effect but sometimes have a deleterious effect on homogeneity of the product. (Figs. 1-l, m, n, o.)

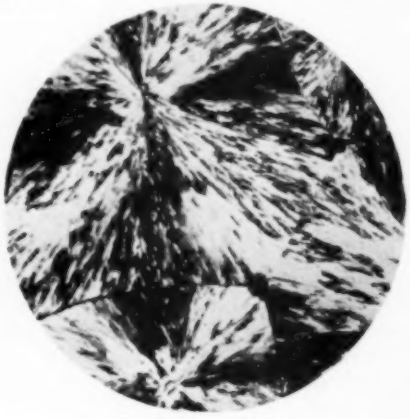
(*i*) Distearates of Polyethyleneglycols 600 and above do not seem to have any effect in reducing crystal size. (Figs. 1-p, q, r.)

(*j*) Whereas the composition of paraffin wax shows evidence of being complex and a mixture of different varieties of crystal forms (a-e, h-n, 8-14), evidence collected so far in the case of "Carbowax" compounds indicates that pure samples of "Carbowaxes" crystallize in single habit.

Fig. 1.—Photomicrographs showing the effect of various surface-active agents on the crystallinity of carbowax compounds.



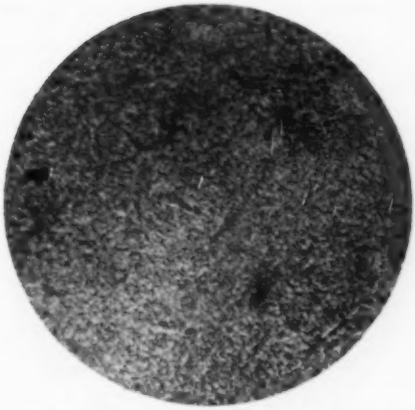
a. Carbowax 1540



b. Carbowax 4000

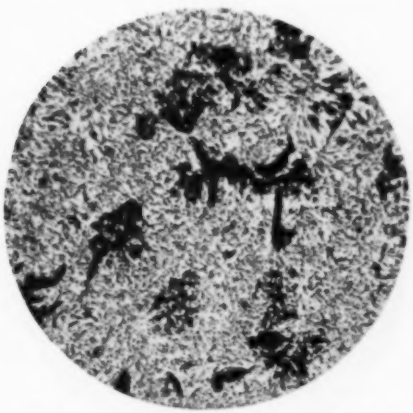
c. Carbowax 6000

d. Carbowax 4000 with 10% water

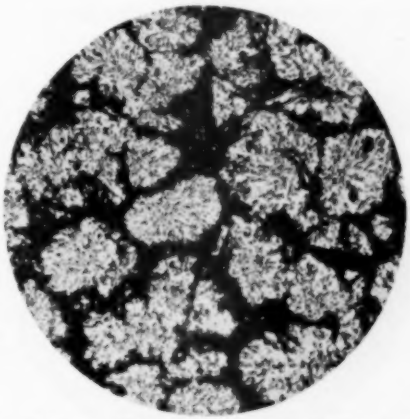


e. Carbowax 4000 with 10% water and 1% Tergitol 04

f. Carbowax 4000 with 10% water and 1% Tergitol 08



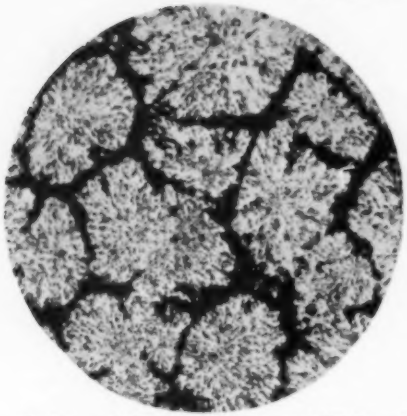
g. Carbowax 4000 with 10% water and 1% Duponol C



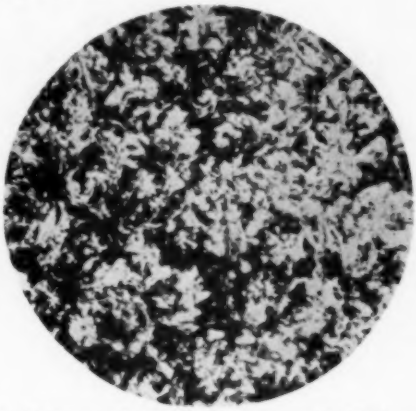
h. Carbowax 4000 with 10% water and 1% Span 40

Fig. 1 (*Continued on page 220*)

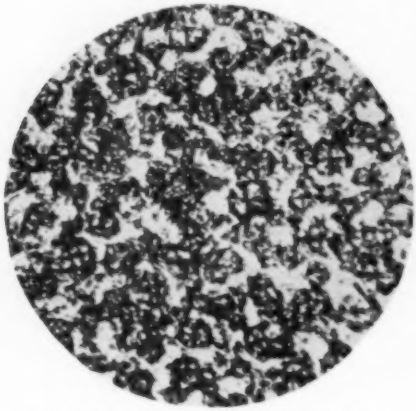
Fig. 1 (*Continued from page 219*)



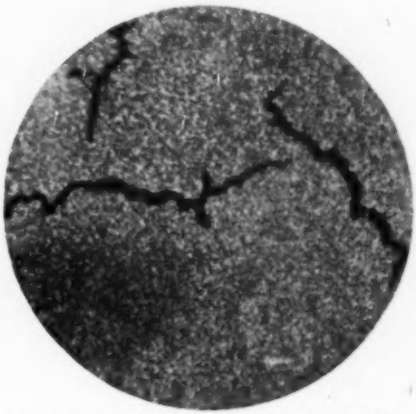
i. Carbowax 4000 with 10% water and 1% Span 60



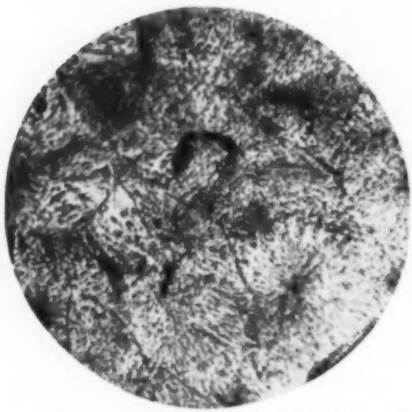
j. Carbowax 4000 with 20% water and 2% Aerosol AY



k. Polyethyleneglycol 400 monostearate

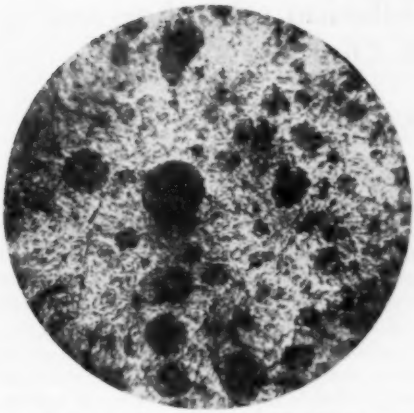


l. Carbowax 4000 with 5% PEG (polyethyleneglycol) 400 monostearate

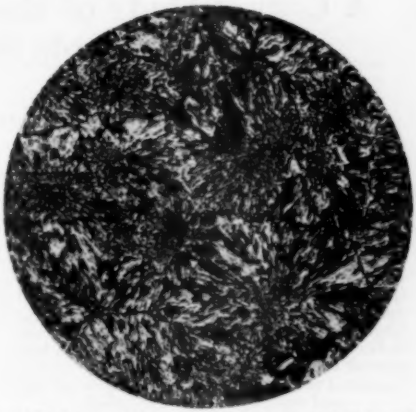


m. Carbowax 4000 with 10% PEG 400 monostearate

n. Carbowax 4000 with 10% PEG 400 distearate



o. Carbowax 4000 with 20% PEG 400 distearate



p. Carbowax 4000 with 10% PEG 600 distearate

q. Carbowax 4000 with 10% Carbowax 1000 monostearate

r. Carbowax 4000 with 10% Carbowax 1540 distearate

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A Critical Study of the Diffusion of Iodine and Sulfathiazole from Ointments*,†

By LAURENCE D. LOCKIE‡ and JOSEPH B. SPROWLS§

Present *in vitro* methods of the study of diffusion of drugs from ointments into gels or solutions are essentially studies of diffusion rates. A physico-chemical approach to the study of diffusion rates is described in this paper. Iodine and sulfathiazole were incorporated into various ointment bases. Types of bases included greases, water-in-oil emulsions, and mucilages. The diffusion into agar gels containing appropriate reagents was recorded at definite time intervals. Diffusion rates, when plotted on graph paper, were found to follow a general pattern, when oil-in-water emulsion and mucilage bases were used.

In recent years, the subject of diffusion from ointment bases has received much attention from both medical and pharmaceutical investigators, particularly with regard to the relative merits of various types of ointment bases as carriers of the newer types of therapeutic agents.

Methods of study have been carried on along two general lines—*in vitro* methods and those involving clinical data. While the final test of the value of any therapeutic agent is its clinical performance, the *in vitro* methods of study are most interesting to the theoretical pharmacist, since they lend themselves to more accurate study and delineation than do the clinical methods. Furthermore, while the *in vitro* methods which have been developed do not closely parallel the actual conditions of use, experience has shown that, for those drugs which are utilized for a purely local effect (for example, the sulfonamide drugs as local disinfectants), the *in vitro* studies have led to the same general conclusions as those which have been reached in the clinical studies.

This has been extensively documented in a recent publication by Huyck, *et al.* (1), in which it was shown that a large majority of the investigators using clinical methods prefer the oil-in-water emulsion type of ointment for the local application of sulfonamides, except in the treatment of burns. *In vitro* tests have indicated that the oil-in-water emulsion type of ointment base releases sulfonamides most readily (1–4).

In vitro studies have primarily been conducted by the agar plate method which was developed by Reddish (5) and described by Ruehle and Brewer (6). This method depends upon the zone of inhibition of bacterial growth which is produced when a given ointment is placed in the center of a Petri dish containing agar which has been uniformly seeded with a bacterial culture. The width of the clear zone (indicating inhibition of bacterial growth) is an indication of the facility of the ointment base to release its content of germicidal agent. This method has gained considerable prominence because of its former acceptance by the Food and Drug Administration as a recognized method for the testing of ointments and other types of germicidal preparations. Other investigators have studied by colorimetric and other methods the diffusion of drugs from ointment bases into agar gels, saline solution, blood serum, and water.

OTHER METHODS

The method developed by Waud and Ramsay (3) and later used by Huyck, *et al.* (*loc. cit*), was as follows: "A 4% solution of agar in Ringer's solution was prepared, and to this was added Ehrlich's solution, which in contact with sulfonamides turns red. After being placed in test tubes and allowed to gel, a layer of the vehicle containing the sulfonamide was placed over the gel, and the extent of the diffusion measured at definite intervals."

Bandelin and Kemp (2) measured the diffusion into saline solution and serum by the following method: "Four grams of the ointment was applied evenly to the inside surface of a 6- x 1-inch test tube, using a glass rod to spread the ointment in a uniform layer with a circular motion. Twenty-five cubic centimeters of saline or serum were then pipetted into the tube (capacity 60 cc.) and the tubes then placed in an incubator at 37.5° for varying lengths of time. Five tubes were thus prepared for each ointment, and one taken for analysis after two, four, six, eight, and twenty-four hours' incubation. The contents of the tube taken for analysis were poured carefully into another tube and agitated by inverting three times. An aliquot of this, taken for analysis, was used directly, or after appropriate dilution, depending upon the relative strength of the drug in the liquid. The amount of sulfonamide in the aliquot was determined colorimetrically by the method of Bratton and Marshall (7), using an electrophotometer and a filter with a maximum transmission at 525 mμ." They also checked their results by means of

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the F. D. A. agar cup plate method, a modification of the agar plate method previously described, wherein 1.5-cm. cups are cut in the agar layer in the Petri dish, and the ointment to be tested is placed within the cup.

R. W. Howard (4) spread the ointment to be tested on a watch glass and placed this in a covered jar containing 100 cc. of water. The jar was incubated at 37.5°. Then, at intervals of six, twelve, twenty-four, forty-eight, and ninety-six hours, 1-cc. samples were withdrawn and assayed by the Bratton and Marshall method.

It is the authors' thesis that since both of these general methods are based upon the diffusion of drugs into gels or solutions, the study of ointment bases by *in vitro* methods is essentially a study of diffusion rates. We therefore believe that a physicochemical approach to the study of ointment bases would be of value. Since the germicidal or therapeutic value of the drug itself can be evaluated by separate means, a measurement of the ability of an ointment base to release its drug content would seem to be an important indication of its potential value.

This statement, of course, gives no consideration to the emollient or protective value which a base might possess of itself. This is often of considerable importance, sometimes sufficiently so as to overshadow other considerations. In this study we are concerning ourselves with those instances in which the release of active ingredients from the ointment base is of primary importance.

EXPERIMENTAL

The drugs selected for these tests were iodine and sulfathiazole, since test solutions which show a color change and can be incorporated in an agar gel are readily available. Ehrlich's paradimethylaminobenzaldehyde solution gives a bright yellow color with sulfathiazole, and starch was used to show the penetration of the iodine. Sulfathiazole was used in the ointments rather than the sodium salt, since Waud and Ramsay (3) have observed that the diffusion of the sodium salt is only slightly faster than that of the sulfathiazole itself.

In preparing the iodine ointments, a solution of iodine was prepared, using 100 Gm. of iodine, 100 Gm. of potassium iodide, and distilled water to make 200 cc. The proper amount of the solution to obtain the desired amount of iodine in the base was then incorporated in the base.

Ointment bases were selected from the following types: (*a*) grease bases, (*b*) water-in-oil emulsion bases, (*c*) oil-in-water emulsion bases, and (*d*) mucilage bases.

A larger proportion of oil-in-water emulsion bases was included since the formulas for these differ widely, and the previous investigators have shown that diffusion from the other types is limited. Some of each, however, were included for purposes of comparison. A further comparison was provided for by using an aqueous solution containing the same concentration of drug as was contained in the ointments. Sodium sulfathiazole was used in the aqueous solution.

Following is a list of the bases used:

GREASE BASES

Petrolatum, U. S. P. XIII (hydrocarbon base)
Wool Fat, U. S. P. XIII (natural fat containing cholesterol)
Crisco (hydrogenated vegetable oil)
Sulfated Hydrogenated Castor Oil (water-miscible grease)

WATER-IN-OIL EMULSION BASES

Hydrophilic Petrolatum, U. S. P. XIII, with 30% water added
Lanolin, U. S. P. XIII

OIL-IN-WATER EMULSION BASES

Hydrophilic Ointment, U. S. P. XIII
Beeler's Base
Schwartz Base
Vanishing Cream "A"
Vanishing Cream "B"
Anhydrous Chipped Ointment Base (Rare Chemicals)
Carbowax-Petrolatum Base
Aerosol OT Emulsion
Diglycol Stearate Emulsion
Sulfated Hydrogenated Castor Oil (SHCO) Emulsion

The oil-in-water emulsion bases were selected to represent different emulsifiers and also to include formulas which had been suggested by researchers in the field. Hydrophilic Ointment, Beeler's Base, and the Schwartz Base all contain sodium lauryl sulfate; but in the first two the balance of the formula differs, and in the Schwartz formula no water is included. The Vanishing Creams "A" an "B" were taken from the Third Edition of the Pharmaceutical Recipe Book. "A" is a formula containing triethanolamine as the emulsifier, and "B" is a formula using potassium stearate. The Aerosol OT formula was included because it represented a truly sulfonated compound, and the SHCO emulsion to include a sulfated emulsifier. Rare Chemicals Anhydrous Chipped Ointment Base is a mixture of cetyl alcohol, stearyl alcohol, and a polyalkylene derivative of stearic acid.

MUCILAGE BASE

Tragacanth Paste.

This is the tragacanth paste formula from the Third Edition of the Pharmaceutical Recipe Book.

References for the other formulas follow: Beeler's Base, J. AM. PHARM. ASSOC., Practical Edition, **3**, 231(1942); Schwartz Base, *ibid.*, **4**, 111(1943); Aerosol OT Emulsion, J. AM. PHARM. ASSOC., Scientific Edition, **35**, 129–140(1946); SHCO Emulsion, *ibid.*, **30**, 145(1941); and Diglycol Stearate Emulsion, "Glyco Cosmetic Manual," Glyco Products Co., 148 Lafayette St., New York City, p. 62.

Formulas not available from the literature are:

RARE CHEMICALS OINTMENT BASE

Anhydrous Chipped Ointment Base[1]	30.0 Gm.
Distilled Water	70.0 cc.

[1] Rare Chemicals Anhydrous Chipped Ointment Base is a mixture of cetyl alcohol, stearyl alcohol, and a polyalkylene derivative of stearic acid.

Melt the base to 80°, heat the water to the same temperature. Add the water to the base with stirring. Stir until cool.—Rare Chemicals, Inc., Harrison, N. J.

CARBOWAX PETROLATUM BASE

Carbowax 1500	20.0 Gm.
Heavy Liquid Petrolatum	10.0 Gm.
Petrolatum	50.0 Gm.

Melt liquid petrolatum and petrolatum together, add carbowax and stir until cool.—Carbide and Carbon Chemicals Corp., 30 E. 42nd St., New York, N. Y.

PROCEDURE

A 4% agar gel was prepared, with the suitable reagent, and poured into standard 6-in. test tubes When cool, the ointment bases containing the desired strengths of the drug to be tested were superimposed upon the gel in a layer approximately 3 cm. deep. The diffusion was then measured at appropriate time intervals. Since it was desirable to establish the character of the early portion of the curve, several readings were taken during the first day.

AGAR GELS USED

1. For Sulfathiazole

Bacto-agar (Difco)	4.0 Gm.
Ehrlich's Solution	1.0 cc.
Ringer's Solution, U. S. P. XIII	100.0 cc.

The agar was added to the boiling Ringer's Solution and stirred gently until uniform. The Ehrlich solution was added just before congelation and mixed thoroughly.

2. For Iodine

Bacto-agar (Difco)	4.0 Gm.
Starch (Soluble)	0.1 Gm.
Ringer's Solution, U. S. P. XIII	100.0 cc.

The agar and the starch were added to the boiling Ringer's solution and stirred gently until uniform.

The results of the first series of tests conducted are shown in Tables I and II.

DISCUSSION OF RESULTS

The sulfathiazole showed almost no diffusion from grease or water-in-oil emulsion bases. The rate of diffusion was fairly uniform from the oil-in-water emulsion bases and the mucilage base, although the rate from the SHCO emulsion was quite low. This low rate may have been due to experimental error, although in all cases at least two samples were tested, and if the results were not in reasonable agreement, the tests were repeated.

Iodine showed a much higher diffusion rate than did the sulfathiazole from both the oil-in-water emulsion bases and the solution. In this case the carbowax base showed a higher diffusion rate than the other bases, but not as high as the aqueous solution. Some diffusion was also observed from the

TABLE I.—DIFFUSION FROM 10% SULFATHIAZOLE OINTMENTS

Base \ Time in Hours:	1	3	6	24	48	72	96	120	168
	Distance in Millimeters								
Petrolatum	T[a]	T	T	5	5	T	T	..	T
Wool Fat	T	T	T	T	T	T	T	..	T
Hydrogenated Vegetable Oil	T	2	3	T	T	T	T	..	T
SHCO	1	2	3	T	T	T	T	..	T
Hydrophilic Petrolatum (30% Water)	T	T	T	5	5	5	T	..	T
Lanolin	0	T	T	T	T	T	T	..	T
SHCO Emulsion	2	3	4	10	12	14	15	15	..
Schwartz Base	2	4	6	11	20	25	27	30	..
Carbowax Base	5	8	10	18	25	31	35	..	43
Hydrophilic Ointment	4	6	8	14	24	28	30	..	40
Vanishing Cream "A"	4	5	7	15	24	28	30	36	40
Diglycol Stearate	3	5	7	15	25	29	33	39	..
Rare Chemicals Base	3	4	6	11	21	24	28	30	..
Beeler's Base	3	5	7	14	26	29	31	38	..
Aerosol OT Base	3	5	7	14	28	31	36	42	..
Vanishing Cream "B"	4	5	7	12	25	29	31	38	..
Tragacanth Paste	4	5	8	15	23	26	30	..	40
10% Aqueous Solution	5	7	10	21	33	39	43	..	..

[a] T means trace.

TABLE II.—DIFFUSION FROM 5% IODINE OINTMENTS

Base \ Time in Hours:	1	3	6	24	48	72	96	120	168
	Distance in Millimeters								
Wool Fat	0	2	2	3	5	6	6	6	7
Hydrophilic Petrolatum (30% Water)	2	4	5	7	9	10	10	11	11
Hydrophilic Ointment	6	10	13	26	38	45	53	59	69
Vanishing Cream "A"	6	11	14	27	36	44	52	58	70
Tragacanth Paste	7	11	14	27	40	48	56	63	74
Carbowax Base	7	10	17	28	43	55	68	80	End of tube
5% Aqueous Solution	8	13	19	39	55	65	77	86	End of tube

grease bases and the water-in-oil emulsion bases, although it was not large.

When the results shown in Table I were plotted on graph paper, with the distances in millimeters as ordinates, and the time in hours as abscissae, the curves showed the same resemblance to parabolic curves as did those recorded by other investigators.

A study of these curves showed that the points observed at each time interval for the oil-in-water emulsion base ointments vary somewhat, but not greatly. The curves showing maximum and minimum diffusion at each time interval are shown in Figure 1. This presented the possibility that experimental error might account for the variations. It was therefore decided to average all the data for each time interval except those for the SHCO emulsion and the aqueous solution. These average figures were then plotted on graphs, and gave the curves shown in Fig. 2 for the sulfathiazole ointments, and in Fig. 3 for the iodine ointments.

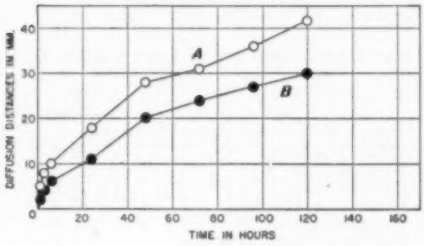

Fig. 1.—Variation in diffusion distances at each time interval.

Data from Table I, with diffusion distances in millimeters as ordinates, and times in hours as abscissae. 10% sulfathiazole ointments.

Curve A. Greatest distance.
Curve B. Smallest distance.

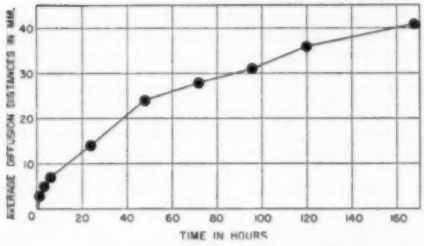

Fig. 2.—Average curve for 10% sulfathiazole ointments.

Diffusion curve for oil-in-water emulsion base ointments with 10% sulfathiazole. Ordinates are average figures for each time interval. Abscissae are times in hours.

Hours	Distance, Mm.	Hours	Distance, Mm.
1	3	72	28
3	5	96	31
6	7	120	36
24	14	168	41
48	24		

Since these were empiric curves, it was decided that a mathematical approach to the problem would be more accurate. Curves of this type are expressed by equations of the type $y^2 = kx$, where the curve passes through the origin. It was assumed for the moment that the curve did pass through the origin, i.e., that the "interface" between the ointment and the agar gel had no effect upon the diffusion time.

Because the rate of change of the slope (coefficient of x, k in the above equation) is not readily determined for curves of this type, advantage was taken of the fact that these curves may be converted to straight lines by setting y^2 equal to another variable, such as z, whereupon the equation becomes $z = kx$ (the equation of the straight line) (8). The slope of this equation then may be readily determined from experimental data by the method of averages (8). This was done in the case of the aqueous solutions of both iodine and sulfathiazole, and for the average data in the cases of the oil-in-water

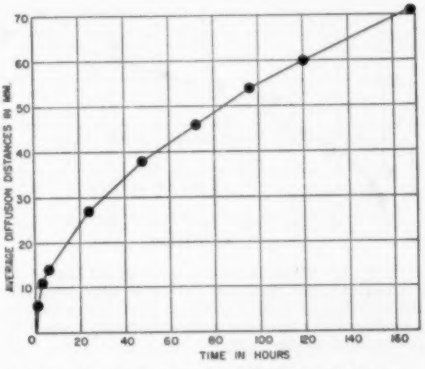

Fig. 3.—Average curve for 5% iodine ointments.

Diffusion curve for oil-in-water emulsion base ointments with 5% iodine. Ordinates are average figures for each time interval. Abscissae are times in hours.

Hours	Distance, Mm.	Hours	Distance, Mm.
1	6	72	46
3	11	96	54
6	14	120	60
24	27	168	71
48	38		

emulsion base ointments of both drugs. The coefficient of x was then rounded off to a convenient number which was well within the probable limit of error with the following results:

Aqueous Iodine Solution.....	60 ($z = 60x$)
Iodine Ointments.....	30 ($z = 30x$)
Aqueous Sulfathiazole Solution.	20 ($z = 20x$)
Sulfathiazole Ointments.....	10 ($z = 10x$)

To further illustrate, the line representing the equation $y^2 = 10x$ was drawn, as in Fig. 4, and the data for 10% sulfathiazole oil-in-water emulsion base ointments plotted to show their relationship to the mathematically determined curve.

Since the coefficient of x expresses the slope of curves of this type, and hence the diffusion rate, it would seem that this number might well be used to express the diffusibility of a substance from a base. Therefore, the coefficient for each oil-in-water emulsion base (and others where it was possible) was determined as above. These coefficients are recorded in Table III.

From Table III the following observations are drawn: The variations from the coefficient for all the oil-in-water emulsion base ointments with sulfathiazole, which was 10.36, are not large enough to prevent showing that they follow a generally similar pattern. The gap between these coefficients and the coefficient of the aqueous solution is much greater than those between the individual coefficients. The coefficients for the carbowax base (13.3) and the Aerosol emulsion (13.3) are high, and the coefficients for the Schwartz Base (7.7) and the Rare Chemicals Base (7.9) are low. Bandelin and Kemp (2) experienced variations in their determinations of about the same magnitude.

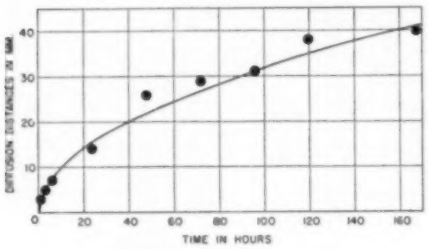

Fig. 4.—Mathematically determined curve.
Curve for the equation $y^2 = 10x$, with points for the diffusion of a 10% sulfathiazole ointment from an oil-in-water emulsion base (Beeler's), shown for purposes of comparison.

DATA

Ordinates, Diffusion in Millimeters	Abscissae, Time in Hours	Ordinates, Diffusion in Millimeters	Abscissae, Time in Hours
3	1	26	48
5	3	29	72
7	6	31	96
14	24	38	120

The general similarity of results obtained with various bases of the same type, and the reproducibility of data indicate that further studies by this method may lead to information from which generalities might be drawn.

For example, the average coefficient of x for 5% iodine in oil-in-water emulsion bases is about 30, while in water solution it is about 60. Also, the average coefficient for sulfathiazole in oil-in-water emulsion bases is about 10, while that for the 10% aqueous solution is about 20. In both cases, the ratio of solution coefficient to oil-in-water emulsion coefficient is 2:1. If further investigation were to indicate that the same relationship holds for other substances, it is possible that the diffusion rate for oil-in-water emulsion bases may be determined by experimental work on the solution.

In the same manner, the relationship between the diffusibility of iodine and sulfathiazole is noted to be 3:1. If such a relationship could be demonstrated to be generally true, then by setting up some substance as a standard, the diffusibility of other substances could be expressed by comparison with this standard. However, much more work must be done before general conclusions such as these can be presented.

BACTERIOLOGICAL TESTS

As a check on the validity of the test-tube method some tests were made by the agar plate method previously described (2). A layer of agar gel, uniformly seeded with *Staphylococcus aureus*, was placed in a Petri dish. One gram of the ointment was placed in the center of the dish. After incubation at 37.5° for twenty-four hours the average width of the clear zone was measured. The results are shown in Table IV where they are compared with the coefficient of x of the bases used.

TABLE III.—COEFFICENT OF x FOR VARIOUS OINTMENT BASES AND SUBSTANCES

Base	Coefficient of x
10% Sulfathiazole Ointments	
SHCO Emulsion	2.8
Schwartz Base	7.7
Carbowax Base	13.3
Hydrophilic Ointment	10.3
Vanishing Cream "A"	10.3
Diglycol Stearate Emulsion	11.4
Rare Chemicals Ointment Base	7.9
Beeler's Base	11.0
Aerosol OT Emulsion	13.3
Vanishing Cream "B"	10.6
Tragacanth Paste	9.8
10% Aqueous Solution	21.2
5% Iodine Ointments	
Wool Fat	0.46
Hydrophilic Petrolatum (30% Water)	1.5
Hydrophilic Ointment	29.0
Vanishing Cream "A"	28.0
Tragacanth Paste	32.4
Carbowax Base	42.8
5% Aqueous Solution	61.2

TABLE IV.—AGAR PLATE TESTS ON OINTMENTS

Base Used	Average Width of Clear Zone, Mm.	Coefficient of x
A. 2% Iodine		
Wool Fat	0.6	0.46
Petrolatum	4.5	...
SHCO	17.0	...
Schwartz Base	27.5	3.1
SHCO Emulsion	35.0	2.3
Diglycol Stearate	50.0	9.4
B. 10% Sulfathiazole		
Wool Fat	Trace	...
Petrolatum	Trace	...
SHCO	4.0	...
Schwartz Base	10.0 partial, a few colonies in zone	7.7
SHCO Emulsion	15.0	2.8
Diglycol Stearate	25.0	11.4

The results of these tests, as in the case of Bandelin and Kemp (2), were in general accordance with the results obtained by the test-tube method. Grease bases showed very small zones of inhibition, while the zones of inhibition were largest in the case of the oil-in-water emulsion bases. The iodine ointments also showed much larger zones than the corresponding zones for the sulfathiazole ointments, although the strength of the iodine ointments was reduced to 2%.

SUMMARY

From the results of the series of tests conducted, the following conclusions may be reached:

1. The diffusion rates of drugs from ointments may be expressed in parabolic curves of the form $y^2 = kx$, in which y represents the diffusion distance in millimeters, and x the diffusion time in hours.

2. These curves may also be expressed in terms of linear equations of the form $z = kx$, in which z represents y^2 of the previous equation.

3. The coefficient of x (k in the foregoing equations) may be used to express the diffusion rate of a substance at a given strength from a given base.

4. The method described seems well adapted to critical studies from which rather broad conclusions may be reached.

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The Antidiuretic Activity of the Posterior Pituitary and Its Quantitative Evaluation*

By K. M. LINDQUIST and L. W. ROWE

Using the method of Edmunds and Cushny as recently modified by Fugo and Aragon (rabbits intravenously), a total of 134 animals, made diuretic by glucose given intravenously, were tested with "Pitressin" and other posterior pituitary fractions to show quantitatively their antidiuretic activity. In most cases the relative antidiuretic activity was in good agreement with the relative pressor values but notably the International Reference Standard was only about 75 per cent as active as the U. S. P. Reference Standard instead of being equally active. One or two modifications are suggested which seem to make the method more accurate than the white mouse and rat methods previously used for antidiuretic assays.

THE METHOD of Burn (1) utilizing white rats injected subcutaneously has been widely used to determine the antidiuretic activity of definitely active posterior pituitary preparations. Considerable personal experience with this method has shown that it is time-consuming and not very accurate unless crossover comparisons are made between standard and unknown with the same groups of rats which makes it still slower in arriving at a result.

The mouse method of Gibbs (2) as modified by Grote, Jones, and Kamm (3) has been used in our laboratories with considerable success but it is also slow and somewhat inaccurate.

EXPERIMENTAL

Recently Fugo and Aragon (4) have suggested a modification of the technique of Edmunds and Cushny (5) which seemed to be both rapid and sensitive. This method utilizes small to medium-sized rabbits which have been given 3 mg. per Kg. of morphine sulfate subcutaneously and 1.5 Gm. per Kg. of urethane intraperitoneally. The jugular vein is then cannulated for infusion of warm saline and the bladder or the urethra is cannulated for collection of the urine. One modification suggested (4) was to give water by stomach tube (5% of the rabbit's body weight) three hours before the actual start of the antidiuretic experiment. We did this for about one-sixth of our 134 experiments. For the next two-thirds we injected the water directly into the stomach of the anesthetized rabbit through a long needle attached by rubber tubing to a 50-cc. burette. The last 20 of our experimental animals received no water orally since it seemed generally to interfere with the uniformity of urine excretion and also delayed the start of the experiment so that fewer comparative tests could be made on the same animal. Another modification (4) is the injection intravenously of 20 to 40 cc. of 20% glucose solution to

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start diuresis. This we found valuable and we standardized on a single injection of 25 cc. of the 20% glucose given intravenously thirty minutes before the first saline infusion of 50 cc. Urine was then collected for forty-five minutes and measured at five-minute intervals, immediately after which the dose of posterior pituitary was injected intravenously and the urine again collected for forty-five minutes. This permitted several injections of sample and standard for comparison in the course of an eight-hour working day.

A typical protocol is given in detail at the top of page 230.

Table I gives the results at a standard "Pitressin" dose versus an unknown dilution.

Table II gives the comparative pressor and antidiuretic activities of 16 different posterior pituitary preparations. The first eight and the last of the samples in the table are regular "Pitressins" or "Pitressin" fractions while the other seven were "Pitressin" Tannate samples that were made soluble with very small amounts of 50% acetic acid and then highly diluted before use. In general the agreement between pressor and antidiuretic activities was very good but it seemed to be a little better in the case of the tannate samples than with the others. Of course this rabbit method tests only the total antidiuretic activity of the tannate samples and tells nothing about their relative repository action just as the dog method gives only their total pressor activity.

Table III gives the comparative results obtained on 21 rabbits between the U. S. P. and the International Reference Standards as well as between the U. S. P. Standard and the standard "Pitressin." The last two appear to be about equal in antidiuretic activity but, except on rabbits Nos. 63, and 66, the International Standard seemed appreciably less active than the U. S. P. Standard. This was quite unexplainable since their antidiuretic activity had been found to be practically equal by the Burn rat method in collaborative tests of the old and new.

TABLE I.—STANDARD "PITRESSIN" (20 PRESSOR UNITS PER CC.) VERSUS AN UNKNOWN DILUTION

Rabbit No.	Wt., Kg.	Cc. Urine, Before	Dose, Unit	Product	Cc. Urine, After	Antidiuretic Effect, %
3	1.9	21.0	0.0020	"Pitressin"	5.0	76.2
		38.0	0.0002	"Pitressin"	36.0	5.3
4	2.5	7.0	0.0002	"Pitressin"	3.0	57.1
		4.5	0.0004	"Pitressin"	2.5	44.4
5	2.2	33.0	0.0002	"Pitressin"	8.5	74.3
6	1.8	11.5	0.0002	"Pitressin"	0.2	98.2
		5.0	0.0001	"Pitressin"	1.5	70.0
7	1.8	14.8	0.0002	"Pitressin"	4.4	70.3
8[a]	1.9	26.6	0.0002	"Pitressin"	10.1	62.1
		24.4	0.0004	Unknown[b]	22.1	9.5
9[a]	2.1	22.1	0.0008	Unknown[b]	20.1	9.1
		22.1	0.0020	Unknown[b]	18.9	14.5
10[a]	2.0	15.6	0.0020	Unknown[b]	6.5	58.4
		25.2	0.0002	"Pitressin"	6.5	74.2
		25.2	0.0020	Unknown[b]	5.4	78.6
11[a]	1.9	11.1	0.0010	Unknown[b]	5.1	54.1
		47.9	0.0002	"Pitressin"	35.3	26.3

[a] In this table the last four rabbits were used (a total of 9 doses) to show that the unknown was relatively weak—certainly less than 50% of the standard on rabbit No. 8 and much more than 20% of the standard on rabbit No. 11. This result of 30% to 40% agrees quite well with the actual dilution of 40%.

[b] The unknown tests less than 50% and more than 20% of the standard "Pitressin." Actually it was made 40% of the standard.

TABLE II.—COMPARATIVE ANTIDIURETIC AND PRESSOR ACTIVITIES OF DIFFERENT "PITRESSINS"

Sample No.	Rabbit Nos.	Antidiuretic Activity	Pressor Activity
522X2A	15 and 21	0.4 Unit/cc.	0.35 Unit/cc.
522X2B	16, 22, 26, 30	2.0 Units/cc.	2.0 Units/cc.
522X2C	18	1.0 Unit/cc.	1.0 Unit/cc.
522X2D	19 and 23	> 0.3 Unit/cc.	0.3 Unit/cc
P 228	41, 43–47	0.005 Unit/cc.	< 0.05 Unit/cc.
P 229	36–40	1.5 Units/cc.	0.6 Unit/cc.
102459	51, 54, 59	14.0 Units/cc.	14.0 Units/cc.
102460	48, 49, 50	15 to 18 Units/cc.	11.0 Units/cc.
X2621	99, 100, 101	4.0 Units/cc.	6.0 Units/cc.
X2622	96, 97, 98	4.8 Units/cc.	4.8 Units/cc.
X2623	90 to 95	3 to 4 Units/cc.	4.8 Units/cc.
X2417	104, 106, 109 110–113	3.3 to 5.0 Units/cc.	5.0 Units/cc.
106294	102, 105, 107 109, 111, 112	4 to 5.5 Units/cc.	5.5 Units/cc.
86953	119–123	6.0 Units/cc.	6.0 Units/cc.
101686	119–123	5 to 6 Units/cc.	6.0 Units/cc.
522X14E	124–131	0.8 Unit/cc.	0.8 Unit/cc.

[1] "Pitressin" will be used throughout to designate the trade-marked name of the commercial product.

TABLE III.—INTERNATIONAL STANDARD, U. S. P. STANDARD AND "PITRESSIN" STANDARD

Rabbit No.	Wt., Kg.	Cc. Urine, Before	Dose, Unit	Cc. Urine, After	Antidiuretic Effect, %
55	2.6	11.0	0.0002 Pitr.	5.7	48.2
		38.5	0.0002 U. S. P.	15.6	59.5
56	2.0	13.0	0.0002 Pitr.	5.0	61.5
		21.8	0.0002 U. S. P.	14.0	35.8
57	1.9	30.3	0.0002 U. S. P.	14.3	52.8
		81.9	0.0002 Pitr.	24.2	70.5
61	2.0	44.1	0.0002 Int.	9.7	78.0
		79.7	0.0002 U. S. P.	6.8	92.5
63	2.0	18.5	0.0002 Int.	0.4	97.8
		22.3	0.0002 U. S. P.	11.8	47.1
64	2.2	34.6	0.0002 Int.	19.7	43.1
		83.0	0.0002 U. S. P.	35.7	57.0
65	1.8	32.1	0.0002 U. S. P.	2.8	91.3
		60.2	0.0002 Int.	14.1	76.6
66	1.8	9.4	0.0002 U. S. P.	2.6	72.4
		5.4	0.0002 Int.	1.2	77.8
69	2.7	28.7	0.0002 Int.	11.7	59.2
		76.6	0.0001 U. S. P.	62.6	18.3
70	1.6	27.6	0.0002 Int.	18.3	33.7
		39.8	0.0001 U. S. P.	37.3	6.3
71	1.7	34.0	0.0004 Int.	25.0	26.5
		32.5	0.0002 U. S. P.	28.5	12.3
72	2.2	21.8	0.0004 Int.	25.9	None
		25.6	0.0003 U. S. P.	45.7	None
73	1.9	199.9	0.0004 Int.	17.0	14.6
		31.5	0.0003 U. S. P.	24.9	21.0
74	2.0	15.0	0.0003 U. S. P.	10.4	30.7
		18.0	0.0004 Int.	28.3	None
76	2.8	19.6	0.0004 Int.	13.4	31.6
		45.9	0.0003 U. S. P.	29.8	35.1
77	1.8	39.7	0.0004 Int.	26.7	32.7
		29.7	0.0003 U. S. P.	21.2	28.6
		58.4	0.0003 Int.	57.0	2.4
78	2.0	11.7	0.0003 U. S. P.	4.6	60.7
		7.7	0.0004 Int.	5.9	23.4
		5.7	0.0003 U. S. P.	7.4	None
81	2.1	17.5	0.0003 U. S. P.	11.6	33.7
		15.7	0.0004 Int.	14.1	10.2
		12.4	0.0003 U. S. P.	10.9	12.1
82	1.8	19.8	0.0003 U. S. P.	23.1	None
		30.0	0.0004 Int.	34.5	None
84	1.8	6.2	0.0003 U. S. P.	5.0	19.4
		5.9	0.0004 Int.	6.6	None
88	2.0	17.5	0.0003 U. S. P.	11.5	34.3
		15.4	0.0004 Int.	14.8	3.9
		14.1	0.0004 U. S. P.	5.0	64.5

International Standards when the new lot was tried out before replacing the old (6). At that time the antidiuretic activity of the U. S. P. Reference Standard was not tested by us at least, but presumably when it was first set up by the U. S. P. Committee its various types of activity must have been checked against that of the International Reference Standard. Rabbits Nos. 69, 70, and 71 showed the International to be more than 50% as active as the U. S. P. Standard and Nos. 76, 77, and 81 showed its activity to be about 75%. It was therefore concluded that the antidiuretic activity of the present International Reference Standard is about 75% of that of the U. S. P. Reference Standard as determined by this rabbit method.

Table IV gives results on 6 rabbits, but only 4 were of any value in showing the effect of varying doses of the same product (the U. S. P. Standard Extract) on the same rabbit. While a dose of 0.00010 unit will give a definite effect especially if given first, the 0.0002 and 0.0003 unit doses are better adapted for use in quantitative assays as shown by rabbit No. 116. The 0.0004-unit dose is apparently too large although rabbit No. 117 shows a nice difference between doses of 0.0003 and 0.0004 unit. In general 4 doses given to the same rabbit are too many to give reliable results but 2 and sometimes 3 doses act quite well on the same animal.

Our results confirm the desirability of giving a large dose of glucose intravenously to start a diuresis (4) and also of giving 50-cc. intravenous saline infusions frequently but at regular intervals. After using water given directly into the stomach in fully 80% of our experiments we found that the omission of this procedure gave more accurate and more rapid comparative results. It is therefore recommended that this modification suggested by Fugo and Aragon (4) be omitted since it is not necessary if saline infusions are given to maintain diuresis.

SUMMARY AND CONCLUSIONS

1. The intravenous rabbit antidiuretic method for the evaluation of the antidiuretic activity of

A TYPICAL PROTOCOL IN DETAIL

Rabbit No. 121, wt. 1.7 Kg. March 3, 1948
3 mg./Kg. Morphine Sulfate subcu. at 8:30 a. m.
1.5 Gm./Kg. Urethane intraper. at 8:30 a. m.
25 cc. of 20% solution of Glucose intravenously at 9:30 a. m.
Doses are in total pressor units given intravenously.

50 cc. Saline I.V. 10:00–10:15		0.0003 Unit 101686 50 cc. Saline 10:45–11:00		50 cc. Saline 11:30–45		0.0003 U. 86953 Saline 12:15–30	
Cc. Urine		Cc. Urine		Cc. Urine		Cc. Urine	
10:05	1.8	10:50	0	11:35	1.0	12:20	1.2
10:10	2.7	55	0.5	40	0.5	25	0.5
10:15	1.2	11:00	0	45	1.0	30	0.5
10:20	0.8	11:05	0	50	0.8		
10:25	1.0	10	1.0	55	1.5		
10:30	0.8	15	0	12:00	0.8		
10:35	0.7	20	0.8	05	2.0		
10:40	0.6	25	0	10	1.8		
10:45	0.6	30	1.0	15	1.2	1:00	2.0
Total	10.2 cc. 100%		3.3 cc. 32.35%		10.6 cc. 100%		4.2 cc. 39.6%

50 cc. Saline 1:00–15		0.0003 Unit U. S. P. Std. Saline 1:45–2:00		50 cc. Saline 2:30–45		50 cc. Saline 3:15–30	
Cc. Urine		Cc. Urine		Cc. Urine		Cc. Urine	
1:05	1.3	1.50	0	2:35	1.0	3:20	0
10	1.0	55	0.7	40	0.5		
15	1.2	2:00	0.8	45	0	30	1.1
20	1.0	05	1.0	50			
25	1.2	10		55			
30	0.8	15		3:00	0.9	45	1.2
35	0	20		05			
40	0	25		10			
45	1.8	30	1.1	15	0.5	4:00	1.5
Total	8.3 cc. 100%		3.6 cc. 43.4%		2.9 cc.		3.8 cc.

Reduction in urine output to 32.35%, 39.6%, and 43.4% of the preceding normal indicates similar antidiuretic activities for Nos. 86953 and 101686 in direct comparison with the U. S. P. standard extract.

TABLE IV.—EFFECT OF VARYING DOSES OF THE U. S. P. STANDARD ON THE SAME RABBIT

Rabbit No.	Wt., Kg.	Cc. Urine, Before	Dose, Unit	Cc. Urine, After	Antidiuretic Effect, %
115	1.9	13.5	0.0002	14.6	None
		15.8	0.0001	15.9	None
		16.0	0.0002	30.9	None
116	2.0	22.7	0.0002	19.7	13.2
		38.6	0.0003	23.1	40.2
		35.1	0.0002	31.9	8.1
		14.2	0.0003	10.4	26.7
117	2.2	14.9	0.0004	6.4	57.1
		29.1	0.0003	45.1	None
		53.6	0.0004	37.0	31.0
		26.3	0.0003	14.9	43.3
118	2.0	26.5	0.0003	23.7	10.5
		47.7	0.0004	51.3	None
		61.1	0.0004	48.9	20.1
		19.3	0.0001	1.3	93.3
132	1.9	37.8	0.0002	28.2	25.4
		48.0	0.0003	34.3	28.5
		54.8	0.0004	49.4	10.0
		3.9	0.0001	1.8	53.8
133	1.5	7.0	0.0002	2.3	67.2
		3.0	0.0003	2.6	13.3
		4.1	0.0004	3.2	21.9
		10.6	0.0001	7.0	34.0
134	1.8	8.5	0.0002	8.2	3.5
		8.6	0.0003	20.4	None
		21.6	0.0004	35.8	None

posterior pituitary preparations has been found to be more accurate and rapid than the previously used rat and mouse methods.

2. Certain modifications such as glucose and saline intravenously are valuable and are recommended but the omission of the oral water dosage with its three-hour delay is desirable since it did not seem to contribute to the accuracy or uniformity of our results.

3. The present International Reference Standard while equal to the U. S. P. Reference Standard in oxytocic and pressor activity was found to be only about 75 per cent as active by the rabbit antidiuretic method.

4. Two and often 3 doses may be directly compared in the same animal for antidiuretic activity, but 4 doses, particularly if the fourth is larger than the others, will usually give an unreliable result.

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The Absorption and Penetration of Therapeutic Agents from a Carbowax Vehicle*

By DONALD B. MEYERS,† M. V. NADKARNI† and LOUIS C. ZOPF‡

The penetration of potassium iodide and phenolsulfonphthalein from "Carbowax" vehicles through the intact skin of albino rats has been studied. A change in rate and amount of penetration caused by the addition of silica, cetyl alcohol, wool fat, petrolatum, and "Tergitol" was demonstrated. Addition of cetyl alcohol and silica markedly reduced the absorption of the dye and the potassium iodide while the addition of petrolatum and wool fat caused a small decrease. Tergitol had little influence. A study also was made on the release of antiseptics from the same vehicles using a modified F. D. A. agar cup method. Absorption of phenolsulfonphthalein from the Carbowax base through the intact human skin was demonstrated clinically.

IT HAS BECOME well recognized that penetration and absorption of therapeutic substances from topical applications vary considerably with the type of vehicle employed. In recent years, many workers have met with success in preparing ointment bases which enhance the therapeutic activity of incorporated medicinal agents through increased absorption and penetration. The use of these newer type vehicles necessitates a study of the measurement and control of this increased activity.

Most therapeutic agents are not readily absorbed by the skin when they are incorporated in a base composed of fats, petrolatum, waxes, and oils. Previous investigators (1–4) have reported that the efficiency of antiseptics in oleaginous bases is much diminished or entirely absent. The addition of water and the presence of oxycholesterol greatly improve the antiseptic value of this type of base (5). This germicidal activity is further enhanced in some of the newer water in oil or oil in water emulsions such as Hydrophilic Ointment, U. S. P.

Anhydrous Carbowax ointment bases which have proved practical as vehicles for antiseptic agents have recently been developed in our laboratories and by other investigators (6, 7). Absorption and penetration of the therapeutic agent incorporated in this type of base is much greater than from the petrolatum and oily bases.

The advantages of increased absorption and penetration are obvious. Antiseptics are released more readily at the site of application; diffusion of the medicament into the diseased area is more rapid and complete; lower concentrations of active ingredients may be employed to obtain the same therapeutic results; and endodermal treatment through external application is possible. However, drugs incorporated in highly penetrating vehicles may tend to be more toxic or allergenic. Fatalities have been reported in the case of external application of sulfur (8) and of salicylic acid (9). For this reason, it is essential that means of evaluating and controlling absorption and penetration be available.

Many methods with numerous modifications have been suggested to determine the absorption of therapeutic agents from an ointment. Neuroth and Lee (1) have compiled a summary of the procedures used by various investigators. These procedures include both *in vitro* (10–13) and *in vivo* (14–16) methods of determinination. More recent is a method adopted by Clark (17) for testing diffusion of sulfa drugs from various vehicles. Though some correlation may be seen in the results of these workers, much of the literature is contradictory due to the variations in proce-

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‡ Professor in Pharmacy, College of Pharmacy, State University of Iowa.

dures and controls. Evaluations of penetration and absorption from ointment vehicles vary, not only with the method used, but also with the therapeutic agent employed in the experiment. Other factors such as temperature, relative solubility of the therapeutic agent, skin condition, etc., alter results.

The purpose of this study was to establish the comparative ratio of absorption of therapeutic agents from White Ointment, U. S. P., Hydrophilic Ointment, U. S. P. and a simple Carbowax vehicle. Also demonstrated was the alteration of absorption through the addition of other ingredients to the Carbowax base.

The Carbowax vehicle used in this study was a heat stable anhydrous base composed of equal parts Carbowax 4000W and polyethylene glycol 400W. Earlier work had shown it to possess a high index of antiseptic release when the F. D. A. agar cup method of antiseptic evaluation was employed. Our present study demonstrates the means by which, through simple addition of other ingredients, the absorption of therapeutic agents from this base can be altered.

To determine the absorption from these vehicles, a modified variation of the Macht (15) method was employed. In this procedure, the ointment is applied to the intact skin of the experimental animal and the active ingredient from the ointment is collected in the urine. A quantitative analysis for the ingredient is then made. In addition we used, as a basis for comparison, a modified agar cup method and a dye penetration method of our own. A preliminary clinical study was undertaken and an attempt was made to correlate the results.

EXPERIMENTAL

Vehicles employed in the following tests were White Ointment, U. S. P. XIII, Hydrophilic Ointment, U. S. P. XIII, and the simple Carbowax vehicle described earlier. The Carbowax base will be referred to as Base I. Absorption studies were also made from Base I with 2% Tergitol 4, Base I with 2% Silica,[1] Base I with 5% cetyl alcohol, Base I with 10% lanolin and Base I with 10% petrolatum.

Method 1.—This procedure was used to determine the rate of absorption. All test ointments contained 2% of phenolsulfonphthalein. Albino rats, free of any skin abrasions, were used as the test animal. A well-defined area, 3 x 3 in., extending on both sides of the spinal column between the forelegs and tail, was clipped using care not to damage the epidermis. Two grams of the test ointment was applied evenly over this area without unnecessary friction. The treated rats were wrapped with adhesive tape in such a manner that they could neither rub off or ingest any of the application. They were then placed in separate metabolism cages and their urine allowed to collect in graduated cylinders containing a solution of 5% sodium hydroxide. At the end of every hour, the urine was expressed from each rat and the time of the first red coloration of the alkaline solution recorded. The results may be seen in Table I.

TABLE I.—RATE OF ABSORPTION OF PHENOLSULFONPHTHALEIN THROUGH THE INTACT SKIN OF ALBINO RATS FROM VEHICLES LISTED

Vehicle	No. of Rats	Time in Hours of First Positive Test	Time in Hours of All Positive Tests	Mean Time of Positive Tests
White Ointment U. S. P.	6	...	None in 12	...
Hydrophilic Ointment U. S. P.	6	8	12	9.5
Base I	6	3	10	6.7
Base I with 2% Tergitol 4	6	5	10	6.7
Base I with 2% Silica	6	5	11	8.0
Base I with 5% cetyl alcohol	6	6	13	9.7
Base I with 10% lanolin	6	5	11	7.3
Base I with 10% petrolatum	6	5	10	7.3

It is not intended that the data of this test, alone, be considered conclusive, but it does reveal a variation in the average absorption rate from the various vehicles. Absorption from Base I is significantly more rapid than from either Hydrophilic Ointment, U. S. P. or from White Ointment, U. S. P., the latter giving negative results. Addition of Tergitol 4 to Base I, which might be expected to increase the absorption rate, did not show such results. Silica and cetyl alcohol added to Base I caused a marked retardation of absorption. These results also indicate that the addition of a small amount of petrolatum or wool fat (up to 10%) does not significantly retard the rate of absorption. The addition of petrolatum might prove advantageous for increasing heat stability without lowering the activity of the base.

Method 2.—In the second method, the procedure is similar to that of the first. The applications employed in this test were 10% potassium iodide ointments using the same vehicles as before. Five grams of the ointment was applied to the clipped area of rats which were then wrapped and placed in metabolism cages as in the previous experiment. The urine was collected at the end of six-, twelve-, and twenty-four hour periods, and analyzed for potassium iodide. The results in milligrams can be seen in Table II.

All results obtained from Method 2 can be correlated reasonably with those obtained from Method 1 with the exception of the ones obtained from Base I with 10% lanolin. In this series of experiments, the quantity of potassium iodide absorbed varied inversely with the time required for penetration of the dye as revealed by the first experiment. The Carbowax base with added wool fat gave unexplainable high absorption of potassium iodide. Previous workers have been unable to agree on how the addition of lanolin to vehicles effected absorption. Luff

[1] Microfine silica supplied by the Linde Air Products Company New York.

(10) and Sauerland (18) show decreased absorption from wool fat containing bases while Breslauer (11) and Brown and Scott (14) conclude that the addition of lanolin to vehicles causes an increased absorption.

TABLE II.—AVERAGE NUMBER OF MILLIGRAMS OF KI FOUND IN THE URINE OF RATS AFTER APPLICATION OF 5 GRAMS OF 10% POTASSIUM IODIDE OINTMENT WITH THE VEHICLES LISTED

Vehicle	No. of Rats	Mg. of KI Excreted in Hours 0–6	6–12	12–24	Total
White Ointment U. S. P.	6	0	0.7	9.1	9.8
Hydrophilic Ointment U. S. P.	6	8.2	10.3	15.6	34.1
Base I	6	19.7	20.2	44.7	84.6
Base I with 2% Tergitol 4	6	14.7	30.2	43.5	88.4
Base I with 2% Silica	6	4.6	9.7	23.1	37.1
Base I with 5% cetyl alcohol	6	6.0	7.1	19.3	32.4
Base I with 10% lanolin	6	21.3	23.3	70.6	115.2
Base I with 10% petrolatum	6	13.6	26.9	38.4	78.9

Absorption of potassium iodide from Base I was eight times as great as from White Ointment, U. S. P. and more than twice that shown by Hydrophilic Ointment, U. S. P. The results also show that absorption from the Carbowax vehicle (Base I) may be reduced by 50% through the addition of 2% silica or 5% cetyl alcohol. As in the first method, these results also show that the addition of 10% petrolatum does not retard absorption significantly from Base I.

Method 3.—The third method employed was an agar cup method. This procedure was used to see how well the results of an *in vitro* test of this type correlated with the *in vivo* tests of Methods 1 and 2.

Melted agar media were inoculated with a strain of *Staphylococcus aureus*, poured into agar plates and allowed to cool. A hole, 15 ml. in diameter, was cut out of the center of the agar with a sterile test tube. Two grams of the ointment containing a germicidal agent was placed into the cavity with a specially constructed syringe, so that it just touched the agar. The plates were then incubated at 35° for thirty-six hours and the zones of inhibition measured. The measurement recorded was the distance in millimeters from the ointment to the inside of the ring of bacterial growth.

Three different series of plates were made, Series A—5% resorcinol; Series B—Ceepryn 1:1000; and Series C—5% sulfathiazole. As Ceepryn loses its antiseptic activity in the presence of a strong anionic compound, results of Ceepryn inhibition were not obtained with Hydrophilic Ointment, U. S. P. which contains sodium laurel sulfate or with Base I containing added Tergitol 4. It has been brought to our attention that Quisno, Gibby, and Foter (19) have demonstrated that agar reduces the germicidal potency of quaternary ammonium salts. It is possible, therefore, that the limited zones of inhibition indicated with Ceepryn are in part due to the inhibition caused by agar.

Table III shows the results obtained from Method 3.

TABLE III.—AVERAGE WIDTH IN MILLIMETERS OF ZONES OF INHIBITED BACTERIAL GROWTH ON AGAR PLATES INOCULATED WITH *Staphylococcus aureus* AROUND THE OINTMENTS LISTED

Vehicle	No. of Plates	Antiseptic Agent: 5% Resorcinol, Series I	1:1000 Ceepryn, Series II	5% Sulfathiazole, Series III
White Ointment U. S. P.	3	0	0	5.0
Hydrophilic Ointment U. S. P.	3	6.0	...	10.3
Base I	3	6.3	5.0	16.0
Base I with 2% Tergitol 4	3	6.0	...	15.6
Base I with 2% Silica	3	4.3	3.0	4.3
Base I with 5% cetyl alcohol	3	8.6	2.0	9.6
Base I with 10% lanolin	3	5.6	4.6	7.0
Base I with 10% Petrolatum	3	7.0	2.6	7.0

A reasonable degree of correlation is evident between the results of the agar plate method and the results of the *in vivo* methods. High results were obtained from resorcinol in Hydrophilic Ointment, U. S. P. and in Base I with cetyl alcohol. This may be explained by the increased solubility of the resorcinol in the stearyl alcohol of the Hydrophilic Ointment and in the cetyl alcohol of the Carbowax base. Lower results were obtained from Base I with lanolin than might be expected from the results of Method 2.

Method 3 is easier to use and less time consuming than Methods 1 and 2 and does not introduce the factor of individual animal variation. However, assuming that Method 2 is the most reliable and most nearly approaches actual clinical results, there are some occasions when the third method gives erroneous results such as those experienced with the resorcinol. There is not sufficient variation in penetration time to make Method 1 a reliable criterion of absorption.

In an initial clinical study, we were able to show absorption of phenolsulfonphthalein through the human skin from the Carbowax vehicle. Five grams of Base I containing 8% of the dye was applied to the patient's side and covered with a plastic bowl. Marked coloration from the dye was obtained in the alkalinized urine of all twelve patients tested, the concentration varying with the condition of the skin. Further clinical evidence is being compiled to substantiate the results of the other experimental data.

By combining the results of all the tests employed, the following conclusions may be drawn.

(1) Absorption of the therapeutic agents employed in this study is better from the Carbowax vehicle than from Hydrophilic Ointment, U. S. P., absorption from Hydrophilic Ointment being much greater than from White Ointment, U. S. P.

(2) Absorption of phenolsulfonphthalein and potassium iodide from a Carbowax base of this type

can be markedly reduced by the addition of 2% silica or 5% cetyl alcohol.

(3) The addition of a wetting agent such as Tergitol 4 is of little value for increasing absorption of phenolsulfonphthalein or potassium iodide from an anhydrous Carbowax base.

(4) Up to 10% of petrolatum may be added to the Carbowax vehicle without noticeably affecting the penetration rate of phenolsulfonphthalein or potassium iodide.

(5) The absorption results obtained by the addition of lanolin to the Carbowax base vary with the method.

SUMMARY

(1) The penetration and absorption of therapeutic agents from White Ointment, U. S. P., Hydrophilic Ointment, U. S. P., and from a Carbowax Base have been studied comparatively using three methods.

(2) A change in absorption value from the Carbowax base after addition of Tergitol 4, silica, cetyl alcohol, petrolatum, and wool fat has been demonstrated.

(3) Methods of retarding the absorption of phenolsulfonphthalein or potassium iodide from the Carbowax base by simple addition of cetyl alcohol or silica have been pointed out.

(4) Further clinical studies are in progress to learn which of the three methods outlined most nearly coincide with clinical findings.

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Book Reviews

Kommentarer Till Svenska Farmakopén, 1946. By WOLMER BONDESON, *et al.* Published by the Apotekarsocietetens Direktion, Stockholm, Sweden, 1947. 552 pp. 14.5 × 21 cm.

Evidence on all sides points to the fact that pharmacopœias all over the world have the same trend toward the inclusion of more potent and specific medications, the only difference being one of degree. Another important trend is the constant improvement of manufacturing methods leading to smaller amounts of impurities, which of course demands more sensitive means of detecting and determining them.

All of these trends lead up to a need, on the part of pharmacists and those scientifically trained persons who use pharmacopœias only occasionally, for adequate commentaries which explain the bases for these tests and standards. All too often when such commentaries are consulted, the reader finds a great deal of data which are useful concerning pharmacology, posology, and toxicology, but frequently only a limited amount of information on the chemistry involved in the standards.

This commentary on the Swedish Pharmacopœia of 1946 is devoted almost entirely to a discussion of the chemistry involved in this Pharmacopœia. Although the difference between Swedish and American standards is frequently rather great, this book will prove to be quite useful to anyone for whom the language is not a handicap.

Reference Book of Pharmaceutical Registration and Label Requirements in Latin America. Compiled by ALBERT AVIGDOR. James A. Marshall, Ltd., 47 West 56th Street, New York, 1948.

This handy little book gives, in outline form, the registration and label requirements for pharmaceuticals in each of the Latin American countries. The arrangement is alphabetical by country.

Under each country, the first page is a data sheet where the user of the book may record such useful information as consular fees for certification of documents, exchange rate, and names and addresses of firms handling registration matters.

The second page deals with Board of Health requirements and tells at a glance which products must be registered, how samples must be submitted how labels must be submitted, how packages are to be registered, all data and documents to be submitted, fees, validity, and prerequisites.

The third page, under each country, is concerned with label requirements. On this page will be found a capitulation of all statements required on labels.

The same plan of presentation is used for each country. The book has a loose-leaf form so that it can be modernized by means of a subscription system.

Since the author of this book was for many years in charge of the registration and label department of a large international drug firm, the information can

be assumed to be accurate. Certainly it is useful information for those exporting drugs to our southern neighbors.

Technic of Medication. By AUSTIN SMITH. J. B. Lippincott Company, Philadelphia, 1948. xi + 255 pp. 11.5 x 19.5 cm. Price $4.00.

Most pharmacists remember the late Bernard Fantus' book, *General Technic of Medication*, the third edition of which was published in 1938, and have wished for a modern treatise to take its place. Dr. Austin Smith has provided that book in an admirable manner.

Technic of Medication is an exceedingly practical book for physicians and others handling drugs and certain therapeutic appliances. Frequently Dr. Smith followed the same pattern as Dr. Fantus, but always the modern viewpoint is projected. An exemplary balance between therapeutic nihilism and the conservative, scientific use of drugs is maintained throughout. As expected, the physician is constantly reminded to use U. S. P., N. F., and N. N. R. drugs in the interest of sound and economic therapy.

Dr. Smith sympathetically discusses the role of hospital pharmacy. At other points will be found a frank discussion of two very old professional problems—the prescribing pharmacist and the dispensing physician. Dr. Smith rightfully concludes that both of these groups have only a limited place in medical care today. The ownership of prescriptions, the telephoned prescription, prescriptions by mail and other problems of inter-professional relations are discussed in an open and fair manner.

Following chapters on general principles, Dr. Smith takes up oral administration of drugs, parenteral administration, rectal and genito-urinary administration, applications to the skin, and mucous membrane applications. Each of these phases is covered briefly but accurately and practically. A final chapter deals with such practical aids as storage of drugs, physical aids, care of equipment, and removal of stains.

It is unfortunate that Dr. Smith curtailed the section on writing the prescription. If this section were more complete, it would make the book an ideal text in prescription writing for medical students. Helpful as the book is, supplementation is necessary in the classroom in order to teach fundamentals of prescription writing to the completely uninitiated.

Ownership of a copy of *Technic of Medication* should enable one to discuss fluently dosage programs, drug absorption, applications to the skin, the rational choice of a drug, and hundreds of other practical matters. A knowledge of the book's contents should also be helpful in advising patrons about the storage of medicinals, the care of insulin syringes, the administration of drugs to children, and other problems.

The British Pharmacopœia, 1948. Published under the General Council of Medical Education and Registration of the United Kingdom, London. Constable and Co., 1948. xl + 914 pp. 14.5 x 23 cm.

The publication of a pharmacopœia is always an important event to pharmacists and the publication of such an important pharmacopœia as the British is doubly eventful.

Since the last British Pharmacopœia was published in 1932, the character of pharmaceutical progress over the past fourteen years is reflected in the types of admissions. Most of the fresh infusions, 17 tinctures and 4 plasters were relegated to antiquity, while 57 injections, 18 tablets, 10 vaccines, and 9 sex hormones entered the stage.

In six instances the strength of official substances was changed. Of especial significance is the change of hydrogen peroxide solution to 5 to 7 per cent which is about double that of the U. S. P.

The general style and format has been changed to coincide more nearly with that of the U. S. P. Latin titles still take precedence, however.

No very significant changes were made in the inorganic drugs.

As would be expected, the changes in the organic field are too numerous to mention. The newly admitted alkaloids were colchicine, ergometrine maleate, ergotoxine ethanesulfonate, and papaverine hydrochloride.

In the field of local anesthetics, butacaine sulfate, butyl aminobenzoate, cinchocaine hydrochloride, benzyl alcohol, and bromethol (tribromethyl alcohol in amylene hydrate) have been added.

In the arsenicals, the dose of neoarsphenamine has been reduced and carbarsone has been newly admitted.

No new barbiturates were added; one acridine antiseptic, aminacrine hydrochloride; one new sulfonamide, succinylsulfathiazole; and the 10 sulfonamides previously recognized by supplement were added.

In the galenical field many changes were made. Tannic acid ointment, chrysarobin ointment, and hydrous ointment of ammoniated mercury were deleted. New ointment bases, an emulsifying ointment, unguentum emulsificans (emulsifying wax 30, white petrolatum 50, and liquid paraffin 20), and a hydrous emulsifying ointment, unguentum emulsificans aquosum (add 1 Gm. of chlorocresol in 699 Gm. of water to 300 Gm. of the former ointment), were admitted. Cetostearyl alcohol and sodium lauryl sulfate were also admitted.

No new solutions were added and 9 were deleted.

Four new suppositories have been added—bismuth subgallate, cocaine hydrochloride, extract of hamamelis, and the latter plus zinc oxide.

No new syrups were added, but Eaton's Syrup and syrup of ferrous iodide were deleted.

The changes in the vegetable drug field are all relatively minor. Surely no one will miss the few deletions such as tamarind.

It is interesting to note that penicillin is defined as "either the sodium salt or the calcium salt of the antimicrobial acid, which is produced when *penicillium notatum* or related organisms are grown under appropriate conditions or in a suitable culture medium." The bioassay does not seem to be as completely described as that of the Food and Drug Administration of this country. Official penicillin preparations are the injection, oily injection (peanut oil-beeswax), cream, sterilized cream, ointment, ophthalmic ointment, and lozenges.

Under injections, the method of handling "dry-filled ampuls" is unique and may be illustrated by Injectio Hexobarbitoni Sodii. This injection is

defined as "a sterile solution of Hexobarbitone Sodium in Water for Injection which is free from carbon dioxide. It is prepared by dissolving the contents of a sealed container in the required amount of water for Injection immediately before use."

The number of tablets increased from 1 in 1932 to 49 in 1948. In addition to tablets of the Seventh Addendum, 18 new ones were added. Perhaps the most unique feature about the tablet monographs is that in addition to listing "usual sizes," a specific dosage is to be dispensed if the physician does not specify. For example, under Tabellae Acidi Ascorbici it advises, "If the quantity to be contained in a tablet is not stated, tablets containing, in each, 50 mg. shall be dispensed or supplied." In addition weight limitations are placed upon individual tablets based on a percentage of the average of the sample.

All in all, the British have done a commendable job in their Pharmacopœia of 1948 under conditions far more difficult than we have ever experienced. Congratulations from all pharmacists, the world over, are in order.

Aquametry. By JOHN MITCHELL JR., and DONALD MILTON SMITH. Interscience Publishers, Inc., New York, 1948. xi + 444 pp. 15 x 23 cm. Price $8.

During recent months, analytical chemistry has been struggling in the face of the dramatic discoveries of organic and physical chemists to make the scientific public more conscious of its importance. The series on *Chemical Analysis* published by the Interscience Publishers has been of material assistance in that regard. Edited by B. L. Clarke and I. M. Kolthoff and with such men as Ashley, Sandell, Willard, and others as an advisory board, the series on monographs of modern chemical analysis has been welcomed by many. *Aquametry* is Volume V of the series.

The finding of moisture in all sorts of substances has been a very important determination. In many analytical laboratories a drying procedure is probably carried out more frequently than any other test. Oven drying, distillation, and many other techniques have been used for this determination. But it was not until the introduction of the Karl Fischer method in 1935 that a precise chemical method was available.

That the Karl Fischer method is of value is evidenced by the voluminous literature recorded on the subject during the fourteen years since its introduction. However, the application of this method is frequently not easy. To evaluate the method critically in book form provides, therefore, a monograph, exceedingly valuable to analytical and control chemists.

This book on Aquametry, a name coined by the authors, is divided into two parts. Part 1 deals with the specific quantitative determination of water by the Karl Fischer reagent. The nature of the reagent is discussed as well as the general directions for its use in different methods. General applications of the reagent to determine not only moisture, but functional groups, are discussed. The last chapter in the section is given over to the determination of water in commercial organic materials.

Part 2 deals with organic reactions involving the liberation or consumption of water. Here one finds separate chapters on the determination of alcohols, carboxylic acids, amines, peroxides, etc.

A unique and helpful feature of this useful book is a chapter on proposed further studies. Here one finds many desirable projects in both the fundamental and applied fields.

The book is well documented and both subject and author indexes appear to be carefully done. The paper, binding, and general workmanship of this book are of the usual high quality found in this series.

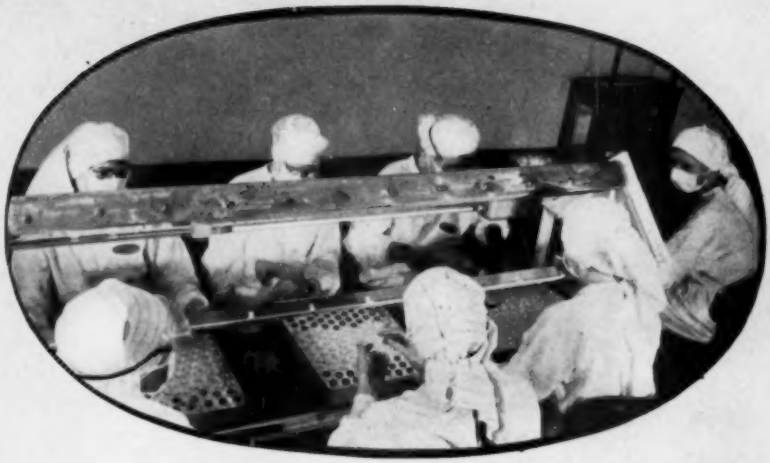
The Literature on Streptomycin, 1944–1948. By SELMAN A. WAKSMAN Rutgers University Press, New Brunswick, N. J. 1948. xv + 112 pp. 15 x 23 cm. Price $3.

Although the isolation of streptomycin was announced in January, 1944, literature has accumulated about 1200 scientific papers during the intervening years. It is fitting, therefore, that the senior discoverer of streptomycin should assemble the references for those interested.

This book opens with a reproduction of the first paper announcing streptomycin as it appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, **55,** 66–69, 1944. This is followed by a selective list of general references on the actinomyces, their antagonistic properties, and on streptothricin. Following this, appear 1171 references to streptomycin arranged in chronological order. An author index and a subject index complete the book.

Die Chemische Industrie und ihre Nebenprodukte. Verlag für wirtschaftsaliteratur G.m.b.H., Burstwiesenstrasse 53, Zürich, 3. 1947. 253 pp. 14.5 x 22.5 cm.

To those who have occasion to use information concerning Swiss chemical firms, this directory will be useful. The directory contains an extensive list of the Swiss chemical and allied industries, arranged according to products. Names and addresses of each firm, leading executives, dates of establishment, banking connections, products and brands, and other essential information are given.



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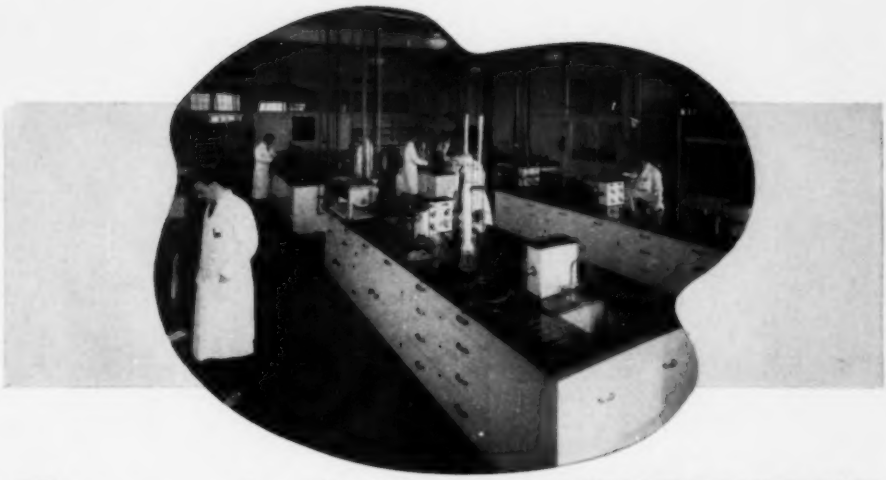

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Kewaunee Laboratory Furniture is built of finest materials by skilled craftsmen in Kewaunee's own plant. Units are streamlined for greatest efficiency and convenience. Kewaunee Metal Furniture is Bonderized *after* fabrication for protection against rusting and chipping.

KemROCK WORKING SURFACES

Working surfaces are of Kewaunee's KemROCK, resistant to acids, alkalies, solvents, abrasion and physical shock.

For a laboratory as modern as "tomorrow," WRITE—

INDUSTRIAL DIVISION

Kewaunee Mfg. Co.

C. G. Campbell, President

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